Lactic acid bacteria as bio-preservatives in bakery – Role of sourdough systems in the quality, safety and shelf life of bread

by

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Abstract

Microbial contamination and survival during storage of bread are a cause of both health concerns and economic losses. Traditional fermentation systems were studied as sources of lactic acid bacteria (LAB) with antagonistic potential against foodborne pathogens and spoilage organisms, with the aim to improve the safety and shelf life of bakery products.

The antagonistic activity of four types of buttermilk (BM) products fermented with *Lactococcus lactis* subsp. *lactis* was evaluated against a number of pathogenic bacteria to select the best fermented-BM for application as bio-preservatives in bread crumpets, showing up to 9 µg/ml of nisin equivalent antimicrobial activity. These food ingredients could be suitable to be used in crumpet formulations,

BM fermented with *Lc. lactis* subsp. *lactis* and nisin influenced the quality and shelf life of crumpets; the pH value and firmness of products with fermented BM was lower and the acidity and springiness was higher than for unfermented BM treatment and control withouth additive. The nisin and fermented BM treatment had beneficial effects on the pore size and colour in comparison with the control, and improved microbial shelf life by 2 days.

Commercial and traditional sourdough and bread samples (n=18) were collected to assess the diversity of LAB strains and potential properties when applied to dough and bread. DGGE followed by sequencing showed that *Lactobacillus* was the predominant genus in the studied sourdoughs. *Lb. plantarum* and *Lb. brevis* strains accounted for 69% of the 32 isolates, out of which 10 were amylolytic and 12 had proteolytic activity. Most were also good acid producers after 24 h at 30°C. Some LAB strains presented a strong *in vitro* inhibitory activity against five indicator strains, showing potential as starter cultures to ferment sourdough.

In subsequent experiments, the properties of 24 sourdoughs were evaluated, and one of them, fermented with *Lb. plantarum* (SIN3) yielded low pH value, high lactic acid production, and suitable microbial growth, and was selected for further bread making performance trials. The bread with fast fermentation and high sourdough concentration (FFHSD) had a lower pH, higher acidity and increased the quality attributes with significantly better shelf life comparing to the other treatments during the storage period. Sensory evaluation demonstrated that fast-fermented breads were more acceptable than the slow-fermented counterparts. Bread prepared with high level (18%) of sourdough fast-fermented with the selected culture (SIN3) had a good eating quality and shelf life. The approach of this study is likely to yield feasible improvements of the current methods of preparation of baking goods.

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List of abbreviations

Abbreviation	Description
AACC	American Association of Cereal Chemists
ANOVA	Analysis of variance
Aw	Water activity
B.	*Bacillus*
BHI	Brilliant Heart Infusion
BLAST	Basic local alignment search tool
BM	Buttermilk
CFU	Colony forming unit
CON	Control group
Con.	Concentration
DNA	Deoxyribonucleic acid
EC	European Commission
EU	European Union
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
FSA	Food Standard Agency
FSAI	Food Safety Authority of Ireland
GRAS	generally recognised as safe
h	hour
HPA	Health Protection Agency
HPLC	High Performance Liquid Chromatography
LAB	Lactic acid bacteria
Lb	*Lactobacillus*
Lc	*Lactococcus*
Lu	*Leuconostoc*
min	Minute
mm	Millimetres

MRS	Man, Rogosa and Sharp
n.d.	Not detected
ng	Nanogram
nt	Nucleotide
OD	Optical density
OTU	Operative taxonomy unit
PBS	Phosphate buffered saline
Pc	*Pediococcus*
PCR	Polymerase chain reaction
ppm	Parts per million
P.	*Pseudomonas*
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscopy
S.	*Staphylococcus*
TA	Titratable acidity
TAE	Tris-acetate- Ethylene Diamine Tetra Acetic Acid
TE	Tris- Ethylene Diamine Tetra Acetic Acid
TPA	Texture profile analyser
TTA	Total titratable acidity
WHO	World Health Organization
UK	United Kingdom
USA	United states of America
µg	Microgram
µl	Microlitre
*L**	Lightness
*a**	Redness
*b**	Yellowness

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Dedication

I would like to dedicate my thesis to my beloved family in particular my father and mother for their patience, without you, I could not reach this level of education and I will always owe you. In addition, I would like to dedicate it to my brothers (Rekar, Rebin and Rawen) who provided the support and encouragement that helped me make it through the tough times.

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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CHAPTER ONE

General introduction and literature review

1.1 Introduction

Lactic acid bacteria (LAB) have a long history of safe use in fermented foods. LAB play an important fermentative role in many foods. They are mainly used in food products due to their contributions to flavour and aroma, increasing shelf life of fermented products and to improve the nutritional and sensory characteristics of the products (Nes *et al*., 1996; De Vuyst and Leroy, 2007; Ravyts *et al*., 2012). Various LAB are used commercially as starter cultures in the manufacture of food products, including dairy products (Salama *et al*., 1995), fermented vegetables (Leisner *et al*., 1996), fermented dough (De Vuyst and Vancanneyt, 2007), alcoholic beverages (Leroy and De Vuyst, 2004), probiotics in animal feeds (Anadón *et al*., 2006), and meat products (Hugas, 1998). Food preservation aims to maintain the quality of raw material and their physical and chemical properties, to improve the quality of the final product and to provide safe and stable products (Brul and Coote, 1999). LAB have an ability to produce antimicrobial compounds such as organic acids (lactic acid and acetic acid), hydrogen peroxide, diacetyl, carbon dioxide and bacteriocins or bactericidal proteins that have been used to preserve food through carbohydrate fermentations and for their effect on texture, colour, taste and smell of food products (Holzapfel *et al*., 2001; Oyetayo *et al*., 2003; Gerez *et al*., 2008). Bacteriocins are produced by several LAB strains and provide an additional hurdle for the growth of spoilage and pathogenic microorganisms. Furthermore, lactic acid and other metabolic products contribute to the sensory properties of a food item. LAB are considered to be safe. They have qualified presumption of

safety (QPS; EU) and are generally recognized as safe (GRAS; USA) status and playing an essential role in food preservation, due to their presence in food and as friendly microflora in the human intestine (Tallon *et al*., 2003; Wessels *et al*., 2004; Bernardeau *et al*., 2008; Settanni and Corsetti, 2008).

An interesting property of some strains of various genera within the LAB group is their ability to synthesise exopolysaccharides (EPS) (Cerning, 1990; Ricciardi and Clementi, 2000). EPS exhibit a positive effect on the texture, mouth-feel, taste perception and stability of fermented foods (Korakli *et al*., 2002; Tieking and Gänzle, 2005).

Since ancient times, many ways of food product preservation have been used by humans such as high concentrations of salt and sugars, using high temperature and chemical addition for preserving food products; some of those methods are still in use. Some of them have negative impacts on the food products, which might affect the quality and nutritional value of the food products and cause health problems (Uhlman *et al*., 1992; Kelly, *et al*., 1996). It is expected that biological preservation methods using metabolites of LAB may enjoy better consumer acceptance than preservation methods that use traditional chemical preservatives. They produce a variety of metabolites which have an antagonistic activity against many microorganisms, including food spoilage and pathogenic microorganisms (Schillinger *et al*., 1996; O'Sullivan *et al*., 2002). They can also improve the quality and safety of food products (Bernardeau *et al*., 2006). LAB have been associated with the human environment and lead to beneficial interactions in food and in the human intestinal tract (Savadogo *et al.*, 2006).

All over the world, very important losses in the baking industry occur due to microbial contamination on bread (Menteş *et al*., 2007). The main reasons for these losses are suitable conditions in bread and other flour based foods for microbial growth, such as water activity (Aw) and pH value. The problems, occurring most frequently in the baking industry, are mould contamination and rope spoilage (Jenson, 1998).

Sourdough is a mixture of flour and water that is fermented by LAB and yeasts. Sourdough fermentation is best known and most studied for its effects on the sensory quality and shelf life of baked goods (Gobbetti *et al*., 1994; Vogel *et al*., 1999; Dy Vuyst and Neysens, 2005). Acidification, activation of enzymes and their effects on the cereal matrix as well as production of microbial metabolites all produce changes in the dough properties and bread matrix that also influence the nutritional value of the products (Di Cagno *et al*., 2003).

The quality of bread is characterised by its flavour, nutritional value, texture, and shelf life (Arendt *et al*., 2007). In the baking industry, these characteristics are improved by addition of bread improvers or enzymes. Alternatively, the addition of sourdough influences all aspects of bread quality and thus meets the consumer demand for a reduced use of additives. As sourdough is an intermediate, not an end product, the microbiological activity has to be determined on the bases of their impact on bread quality. Biochemical changes during sourdough fermentation occur in protein and carbohydrate components of the flour due to the action of microbial and flour endogenous enzymes. The rate and extent of these changes that occur in sourdough fermentation affects the properties of bread such as improving the nutritional value by increasing levels of bioactive compound and inducing mineral bioavailability, producing

flavour volatile compounds, which results in change of the flavour and taste of the final-product, and prolonging shelf life of the bread product through antimicrobial components produced by LAB (Hammes and Gänzle, 1998; Dal Bello *et al*., 2007; Poutanen *et al*., 2009; Galle, 2013).

LAB are also responsible for a range of roots and tubers producing staple foods e.g. cassava fermentation, which might be able to produce antimicrobial compounds which can inhibit the growth of both spoilage and pathogenic organisms, contributing to stability and safety of the fermented product (Holzapfel, 2002). Cassava (*Manihot esculenta* Crantz) is a tropical root crop that serves as food security and income generation for many millions of people in the developing world (Scott *et al*., 2000). Fermentation is an important processing technique used to reduce toxicity and prevent post harvest deterioration of the roots. Cassava fermentation processes can be classified into two main types: solid state, as in gari production, where cassava is grated prior to fermentation and constantly watered during the process; and the submerged process as in fufu or lafun production, where the tubers are cut into chunks and soaked in water for the duration of the fermentation by LAB during which both acidification and softening of the roots take place (Oyewole, 1995; Moorthy and Matthew, 1998).

1.2 The concept of LAB

The term LAB was used for the first time in 1919 by Orla-jensen who studied the characteristics and qualities of this group of bacteria and classified then into four main genera *Lactobacillus*, *Tetracoccus*, *Streptococcus* and *Betacoccus*

(Priest and Campbell, 1999). LAB are a genetically distinct group of bacteria that have same traits, such as: Gram-positive, non-spore forming, rods or cocci, catalase negative, acid tolerant, homofermentative or heterofermentative, lactic acid fermented as the major end product during carbohydrate fermentation, and growth under anaerobic conditions, but also they are aerotolerant (Wessels *et al*., 2004; Axelsson, 2009). The classification of LAB into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, production of lactic acid, ability to grow at different salt concentration, and acid alkaline tolerance (Axelsson, 2009). LAB include a number of bacterial genera: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Lactococcus*, *Vagococcus*, *Oenococcus*, *Enterococcus*, *Aerococcus*, *Lactosphaera*, *Carnobacterium*, *Tetragenococcus* and *Weissella*, but the first four genera are the main group of the LAB (Stiles and Holzapfel, 1997; Carr *et al*., 2002; Hutkins, 2006).

LAB have been isolated from milk and then found in such foods and fermented products as dairy products, meat, vegetables, bakery products and alcoholic beverages (Carr *et al*., 2002). In addition, LAB have been used as a starter culture in the preparation of fermented food products, and they have the ability to develop flavour in these products. For that reason LAB have been used as flavouring and texturizing agents as well as being used in manufacturing and preserving of food products (Richard *et al*., 2006).

There are several potential health or nutritional benefits associated to strains species of LAB. Among these are: improved nutritional value of food, reduced risk of intestinal infections, improved digestion of lactose, potential prevention of some types of cancer, control of serum cholesterol levels and stimulation of the

immune system (Gilliland, 1990). Some of the strains of LAB are considered to be probiotic, which means that they are capable of exerting a beneficial effect on the host. Those must be non-pathogenic and non-toxic, and capable of surviving and metabolising in the gut environmentand also stable and viable after long periods in storage (Khetarpaul, 2005).

1.3 The concept of safety and fermented food

Fermentation is one of the oldest forms of food preservation in the world. The central role of LAB in acid fermentation is now widely acknowledged, and it is accepted that these microorganisms exert beneficial effects through two mechanisms: direct effects of the live microbial cells, known as the 'probiotic effect', or indirect effects during fermentation, where these microbes act as cell factories for the generation of secondary metabolites with health-promoting properties (Hayes *et al*., 2007). Some species of LAB are called beneficial microorganisms which are used in fermentation of various food products such as cheese, yogurt, fermented sausage, pickles, and bakery products, by producing enzymes, flavouring and odorous compounds, acids, and antimicrobial agents. These metabolism products have the ability to extend the shelf life of products, inhibit the growth of spoilage and pathogenic microorganisms, and at the same time they have an effect on retaining the sensory qualities of the product such as texture, flavour, colour and nutritional value (Bernardeau *et al*., 2006; Reis *et al*., 2012). Species that have been used for food fermentations belonging to the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* are shown in the Table 1.1.

Table 1.1: Fermented food products and their related LAB. Adapted from Leroy and De Vuyst (2004) and Kostinek *et al*. (2005)

Fermented food products	**Lactic acid bacteria***
Dairy products:	
Cheese	*Lc. lactis* subsp. *lactis,* *Lc. lactis* subsp. *cremoris,* *Lu. menestroides* subsp. *cremoris*
Butter and Buttermilk	*Lc. lactis* subsp. *lactis,* *Lc. lactis* subsp. *Cremoris* *Lc. lactis subsp. lactis var. diacetylactis*
Yoghurt	*Lb. delbrueckii* subsp. *bulgaricus*, *Strep. thermophilus*
Fermented and probiotic milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus,* *B. lactis, B. bifidum, B. breve*
Kefir	*Lb. kefir, Lb. kefiranofacies, Lb. brevis*
Fermented meats:	
Fermented sausage	*Lb. sakei, Lb. curvatus*
Fermented cereals:	
Sourdough	*Lb. plantarum, Lb. brevis, Lb. farciminis* *Lc. Lactis* subsp. *Lactis*, *Lb. Fermentum* *Lb. Sanfransiscensis, Lb. Brevis, Lb. Amylovorus, Lb. Reuteri, Lb. Pontis, Lb. Panis, Lb. Alimentarius, Weissella cibaria*
Sorghum	*Lb. plantarum*, *Lb. sakei, Lb. curvatus, Lu. Menestroides, Pc. Acidilactici,* *Pc. Pentosaceus*
Fermented vegetables:	
Pickles	*Lb.* plantarum, *Lu. Menestroides*
Fermented olives	*Lb. Plantarum*, *Pc. Acidilactici*, *Pc. Pentosaceus*
Fermented root crops	
Fermented cassava (gari and fufu)	*Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. casei, Lb. fermentum, Lb. reuteri, Lb. delbrueckii, Lb. acidophilus, Lu. fallax, Lu. mesenteroides*
Fermented fish products	*Lb. alimentarius, C. piscicola*

B.=Bifidobacterium Lc.=Lactococcus; *Lb.=Lactobacillus*; *Lu.=Leuconostoc*; *Pc.=Pediococcus*; *Strep.=Streptococcus*; *C.=Carnobacterium*

1.4 Cereal microflora

Cereals are a staple food source for humans because of energy supply and nutrition. Cereals have been used as main staple diet by people of all races (Rosell, 2016). When grains are grown, handled and processed, microbial contamination of cereals can be found at traceable levels to the environment. Contamination of cereal grains is caused by physical and chemical contamination and microorganisms that may come from air, dust, soil, water, insects, rodents, birds, animals, humans, storage and shipping containers, and handling and processing equipment (Smith *et al*., 2004). Environmental factors affecting microbial contamination of cereals can be rainfall, drought, humidity, temperature, sunlight, frost, soil conditions, wind, insect, bird and rodent activity, harvesting equipment, use of chemicals in production versus organic production, storage and handling.

Raw cereals and cereal products contain microflora which include bacteria, yeasts and fungi ranging from 10^4 –10^7 CFU/g, while flour contains 2×10^4 – 6×10^6 CFU/g (De Vuyst and Neysens, 2005). Mesophilic bacteria are mainly found in spontaneously fermented sourdoughs. They include Gram-negative aerobes such as *Pseudomonas* and facultative anaerobes Enterobacteriaceae, as well as Gram-positive LAB which include homofermentative rods (*Lb. casei*, *Lb. coryniformis*, *Lb. curvatus*, *Lb. plantarum*, and *Lb. salivarius*), heterofermentative rods (*Lb. brevis* and *Lb. fermentum*), homofermentative cocci (*E. faecalis*, *Lc. lactis*, *Pc. acidilactici*, *Pc. parvulus*, and *Pc. pentosaceus*), and heterofermentative cocci (*Leuconostoc* and *Weissella*). Also, undesirable *Bacillus cereus*, *Bacillus subtilus*, *Bacillus licheniformis*, *Bacillus mesentericus*, *Staphylococcus aureus* and *Escherichia coli*, as well as other bacteria, may be

present (Galli *et al*., 1987; Corsetti *et al*., 2001). Cereals are considered a good medium for microbial fermentations, because they are enriched with polysaccharides, which are a main source of carbon and energy. Starch is a major polysaccharide in cereals, which can be used by microbes after hydrolysis (Salovaara, 2004). LAB are capable of utilizing starch to ferment cereals (Nguyen *et al*., 2007). Improvement in starch digestibility during fermentation can be related to enzymatic properties of fermenting microflora that brings about the breakdown of starch. The enzymes bring about cleavage of amylase and amylopectin to maltose and glucose (Karovičová and Kohajdová, 2007).

The following yeasts have been reported, either in cereals up to 9×10^4 CFU/g and in flours up to 2×10^3 CFU/g: *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula*, *Torulaspora*, *Trichosporon*, *Saccharomyces*, and *Sporobolomyces*. *Saccharomyces cerevisiae* occurrence in sourdough may be explained by the application of baker's yeast in most daily bakery practice (Galli *et al*., 1987; Corsetti *et al*., 2001). Fungi (up to 3×10^4 CFU/g), especially *Alternaria* and *Fusarium*, as well as *Cladosporium*, *Drechslera*, *Helminthosporium*, and *Ulocladium* are found from the field (dried cereals), while *Aspergillus*, *Wallemia sebi*, *Penicillium*, *Rhizopus, Mucor* and *Neurospora* are found from the storage and bread products (Galli *et al*., 1987; Pateras, 1998; Corsetti *et al*., 2001; Hutkins, 2006).

1.5 Spoilage and pathogenic microorganisms associated with bread and bakery products

The most common and influential factor on bakery products shelf life determination is microbial spoilage in particular mould growth and *Bacillus* sp. (Smith *et al.*, 2004), even when different chemical and physical factors can also reduce.

Fungi are the main cause for spoilage of bakery products, posing a serious economic concern. Moulds involved in spoilage of bakery products mainly include *Eurotium*, *Penicillium* sp., *Aspergillus* sp. *Fusarium*, and *Wallemia sebi* and also *Cladosporium*, *Muco*r and *Rhizopus* especially in warmer climates (Legan, 1993; Gerez *et al*., 2009). Moulds are also responsible for mycotoxin production, a public health issue (Legan, 1993; Nielsen and Rios, 2000). Common bakery products and ingredients' spoilage yeasts belong to *Candida, Pichia*, *Zygosaccharomyces*, *Saccharomycopsis*, and *Debaryomyces* genera (Legan and Voysey, 1991). For example, "chalk mould" is caused by *Pichia butonii* (Pateras, 1998). Baked goods can be protected from fungal and yeast spoilage using LAB with high and wide antimicrobial activities as starters for sourdough preparation (Lavermicocca *et al*., 2000). Gerez *et al.* (2009) reported that some strains of LAB (*Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus reuteri*) showed antifungal activity against *Aspergillus*, *Fusarium*, and *Penicillium*, which are main contaminants in bakery products.

A type of bread spoilage is ropiness, characterised by fruity odour, and then soft and sticky crumb as a consequence of enzymatic degradation of crumb and generation of extracellular slimy polysaccharides (Thompson *et al*., 1993; Pepe *et al*., 2003). Ropiness ocurrs mostly in summer or at hot locations when high

temperature and pH are the environmental factors that increase the growth of bacteria (Saranraj and Sivasakthivelan, 2016) such as *Bacillus* genus mainly *Bacillus subtilis*, and *Bacillus licheniformis*, but *Bacillus cereus*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus clausii* and *Bacillus firmus* have also been implicated (Katina *et al*., 2002; Mantzourani *et al*., 2014). Health problems could be associated to high numbers of *Bacillus subtilis* or *Bacillus licheniformis* in foods which may result in mild food poisoning. To prevent bread ropiness, fermented sourdough with LAB may be effective, because rope-forming bacteria are sensitive to low pH (Hansen, 2012).

Cereal-borne outbreaks involving *Salmonella* sp., *Listeria monoctyogenes* and *Bacillus cereus* have been reported. Pathogenic microorganisms may be introduced into bakery products where ingredients including dairy products, eggs, chocolate, desiccated coconut, and cocoa powder could be the vehicles, with eggs being the most common source of *Salmonella* sp. transfer (Smith and Simpson, 1995). Furthermore, *Clostridium botulinum* is of concern, particularly in high-moisture cereal products which are sealed under modified atmospheres (Smith *et al*., 2004). However, there is limited information about the occurrence of *Clostridium botulinum* in bakery ingredients or end bakery products. There are studies reporting bakery products' contamination with *Clostridium botulinum* type A and B spores, which are ubiquitously observed in soil in agricultural and animal products (Smith *et al*., 2004). Collins-Thompson and Wood (1993) stated that dairy products, which are common on some bakery formulations, have an extremely low incidence of *Clostridium botulinum* spores and are seldomly implicated in botulism outbreaks. Botulism outbreaks have been caused by a variety of fruits and vegetables (e.g. peppers, tomatoes, potatoes, mushrooms,

onions, garlic, olives, and peanuts), which are used in the production of sweet and savoury bakery products (Notermans, 1992). Therefore it is important to evaluate botulism risks in cereal products separately in the components utilised in cereal products (Smith *et al*., 2004). Unfilled pastry products can also undergo spoilage. However, in contrast with bread, when pastries are filled, they can be contaminated by other pathogenic microorganisms such as *Bacillus cereus* and *Staphylococcus aureus*, especially if the filler has been in contact with ingredients such as egg and dairy products (Smith and Simpson, 1995). The most abundant pathogenic bacteria associated to dairy products are mycobacteria, *Brucella sp*., *Listeria monocytogenes*, *Staphylococcus aureus* and *enterobacteria* including toxigenic *E. coli* and *Salmonella* (Brisabois *et al*., 1997). Thus, there is a real concern when using dairy products in bakery products because of the possibility microbial transmission from the dairy product to the end product (e.g. cream filled bread).

1.6 Chemical preservatives

Food additives are defined as chemical substances intentionally added to foods, directly or indirectly, in known quantities, for purposes of assisting in the processing of foods, preservation of foods, or in improvement of the flavour, texture, or appearance of foods (Saltmarsh and Insall, 2013). All food additives are covered by Regulation (EC) No. 1129/2011 of the UK and EU and approved for use in foods and their conditions of use. Chemical preservatives are food additives used to keep foods safe for longer by protecting them against pathogenic microorganisms, and also from spoilage therefore increasing shelf life (Russell, 1991). Many chemicals can kill microorganisms, or stop their

growth, but most of them are not permitted in foods. Chemicals that are permitted as food preservatives by the Food Standard Agency (FSA) in the current EU are propionic acid (E280), sodium propionate (E281), calcium propionate (E282), potassium propionate (E283), sorbic acid (E200), sodium sorbate (E201), potassium sorbate (E202), calcium sorbate (E203) and sodium acetate (E262). Preservation of bakery products commonly involves the use of preservatives such as propionates and sorbates, and sometimes benzoates (Marín *et al*., 2003). Table 1.2 shows the list of permitted preservatives and use as recommended for bakery products with the maximum level of use.

Table 1.2: List of permitted preservatives and use as recommended for bakery products by the European Commission Regulation (EC) No. 1129/2011

E-number	Name	Maximum level (mg/l or mg/kg)	Permitted use in bakery products
E280-283	Propionic acid and propionate	2000	Bread and rolls; energy-reduced bread, partially baked prepacked bread, prepacked rolls, pitta and tortillas Fine bakery wares which category covers sweet, salty products; such as cookies, cakes, muffins, doughnuts, biscuits, pastries, crumpets, pancakes
E200-203	Sorbic acid and sorbates	2000	Bread and rolls; prepacked sliced bread and rye-bread, partially baked, prepacked bakery wares and energy-reduced bread intended for retail sale
		2000	cookies, cakes, muffins, croissants, doughnuts, biscuits, pastries
E262	Sodium acetate	*Quantum satis**	Bread and rolls

* no maximum level is specified, use as much as necesary

Organic acids are such effective food preservatives because, apart from their antimicrobial inhibitory activities, they are also acidulants acidifying and lowering the pH of foods to levels that inhibit bacterial growth (Hinton Jr, 2006). Weak organic acids such as propionic, benzoic, and sorbic acids have been used as food preservatives for a long time, due to their comprehensive antimicrobial activity (Steiner and Sauer, 2003; Plumridge *et al*., 2004) prolonging the shelf life of bakery products (Legan, 1993; Gould, 1996). The antimicrobial activity of these compounds is mostly attributed to undissociated molecules (Zeuthen and Bøgh-Sørensen, 2003). Sorbates (sorbic acid) have many uses because of a milder taste, greater effectiveness, and broader pH range of inhibitory activity in comparison with either benzoate or propionate (Barbosa-Cánovas *et al*., 2003).

Previous *in-vitro* observations have found that calcium propionate, sodium benzoate and potassium sorbate were affective at inhibiting some moulds e.g. *Eurotium*, *Aspergillus* and *Penicillium* isolates from bakery products at pH 4.5 when they were used at a concentration of 0.3% (Marín *et al*., 2002; Guynot *et al*., 2005). Potassium sorbate has been noticed to be effective, even at concentrations as low as 0.03% (Guynot *et al*., 2005).

There are certain harmful adverse effects of using weak organic acids; for example, benzoates have been responsible for worsening asthma, allergic rhinitis, skin rashes, chronic urticaria, hyperactivity and flushing in some people (Kinderlerer and Hatton, 1990; Poulter, 2007; Motala and Steinman, 2008). Despite perceived harmlessness and the sensorial neutrality of sorbic acid (Banerjee and Sarkar, 2004), concerns are linked to situations when it is used together with benzoic acid, which could result in oxidative stress in humans.

Benzoic acid is also generally regarded to be safe, being conjugated in the liver to produce benzoylglycine (hippuric acid), which is then excreted out with the urine. Sorbic acid is also largely excreted, as the oxidation product 2,4-hexadienedioxic acid. The problem is that before these acids are transported to the liver, they will come into contact with epithelial cells of the gastrointestinal tract, but the claimed pro-oxidant negatively effects were uncertain (Piper, 1999; Theron and Lues, 2011). Moreover, concern regarding excessive use of benzoic or sorbic acid which may lead to dangerous side effects such as metabolic acidosis, convulsions, and even hyperpnea in humans (Tfouni and Toledo, 2002; Wen *et al*., 2007). Small amounts of bromate (≤30 mg/kg) may be used in flour or dough during the bread making, however, this can be broken down to bromide during the process as reported by international agency for research on cancer (IARC, 1986). JECFA (1995) reported that the previous acceptable level of treatment of flour for bread making (0-60mg of bromate per kg of flour) has been withdrawn, and the use of potassium bromate as a flour treatment agent is not appropriate, because it strongly irritates the gastric mucous membrane, causing nausea and sometimes vomiting.

1.7 Use of LAB and their metabolites in the food industry as bio-preservatives

There are demands by consumers regarding fresh, healthy and natural food with no chemical preservatives (a so-called “clean label”), with low level of fat, salt, and sugar (Zink, 1997; Brul and Coote, 1999). Consumers are concerned about the presence of artificial chemicals in their foods and prefer natural

compounds (Zeuthen and Bøgh-Sørensen, 2003). Therefore, consumer demands for natural products have encouraged the research on alternative preservatives e.g. safe vegetal and microbial preservation systems. In this respect, LAB are organisms of interest for their bio-preservation potential in different fermented foods as reflected by emerging literature. Hansen (2012) reported that the use of fermented sourdough with LAB is the most promising procedure to preserve bread from spoilage, which is in agreement with the consumer demand for natural and additive free food products. LAB are important microorganisms in the traditional industrial field that can be used for fermentation in food products and are also recognized as healthy and beneficial organisms (Gilliand, 1990). LAB play an important role in the food industry and can be useful as starter cultures for food products (Holzapfel *et al*., 1995; Leroy and De Vuyst, 2004; Mayra-Makinen and Bigret, 2004). LAB produce a wide range of metabolites through fermentation which play a substantial role in inhibiting the growth and proliferation of food spoilage and pathogenic microorganisms (Zottola *et al*., 1994; Cintas *et al*., 1998; Lahtinen *et al*., 2011; Holzapfel and Wood, 2014). There are two main fermentation pathways of glucose in LAB as shown in Figure 1.1. Homofermentative LAB use the Embden – Meyerhof - Parnas pathway to produce more than 85% lactic acid from glucose. Heterofermentative LAB, meanwhile, use the 6P-gluconate pathway or phosphoketolase pathway and produce only 50% lactic acid with an amount of acetic acid, ethanol, and carbon dioxide (Caplice and Fitzgerald, 1999; Zalán *et al*., 2010; Lahtinen *et al*., 2011; Holzapfel and Wood, 2014). There are several studies which indicate closely the effectiveness of LAB and their metabolites as food preservatives (Trias Mansilla, 2008).

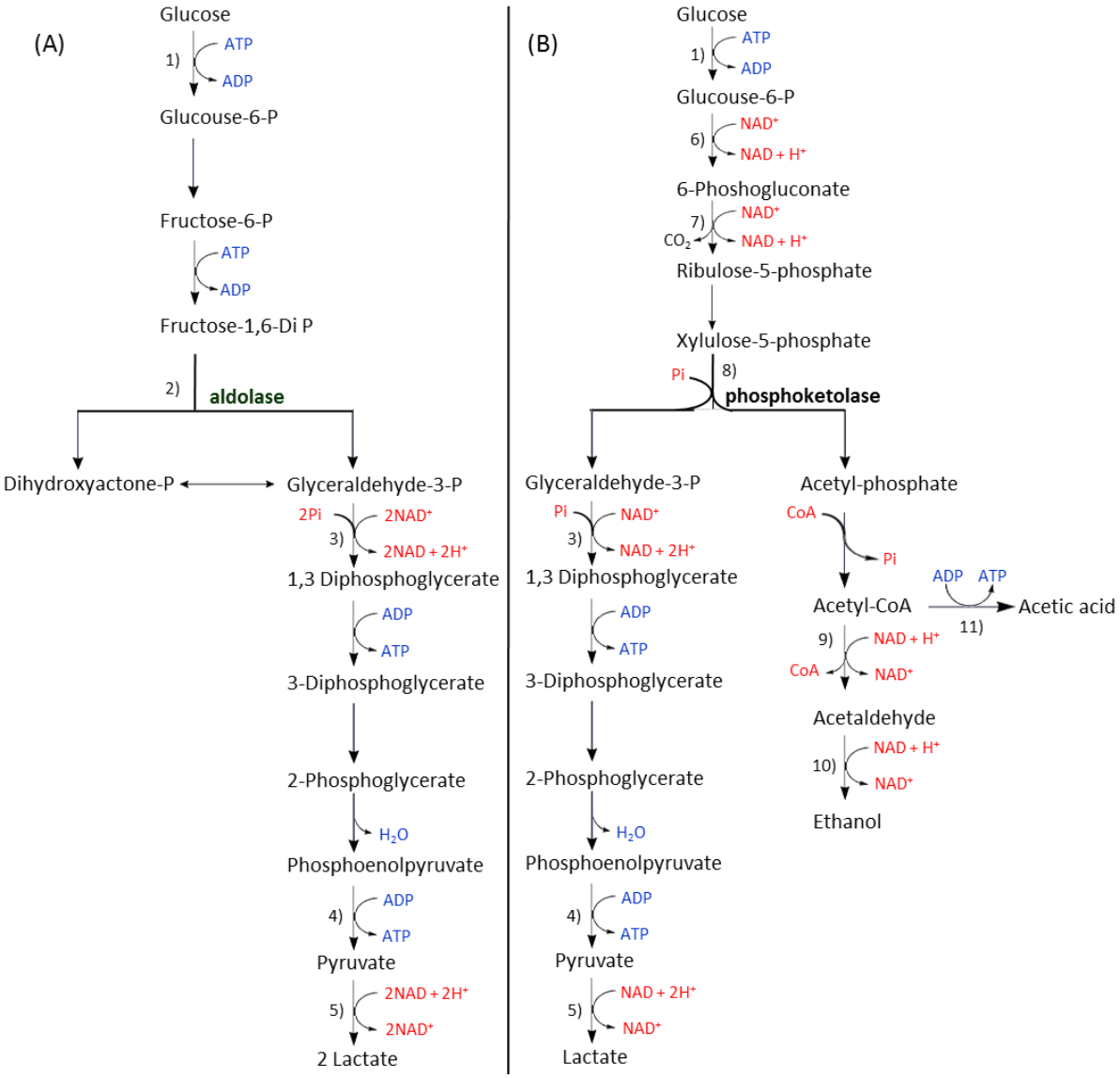


Figure 1.1: Metabolic pathways of LAB: (A) Homo-fermentation (Embden - Meyerhof - Parnas pathway) (B) Hetro-fermentation (6P-gluconate or phosphoketolase pathway). Selected enzymes are numbered: 1) glucokinase; 2) fructose-1,6-diphosphate aldolase; 3) glyceradehyde-3-phosphate dehydrogenase; 4) pyruvate kinase; 5) lactate dehydrogenase; 6) glucose-6-phosphate dehydrogenase; 7) 6-phosphogluconate dehydrogenase; 8) phosphoketolase; 9) acetaldehyde dehydrogenase; 10) alcohol dehydrogenase and 11) acetatekinase (Lahtinen *et al*., 2011; Holzapfel and Wood, 2014)

Several compounds in nature are able to inhibit microorganism growth (Roller, 2003). They can be derived from plants, animals or microorganisms. However, many of them have a limited spectrum of activity and are effective only at very high concentrations. Combining antimicrobial agents is a way to overcome these problems, since a synergistic action may be observed, allowing the use of lower concentrations. At the same time a wider spectra of microbial inhibition can be found.

1.8 Antimicrobial compounds produced by LAB

1.8.1 Organic acids (lactic acid and acetic acid)

LAB produce organic acids during growth; lactic acid and acetic acid are two of the acids that are produced which have an important role in inhibiting the growth of microorganisms (Cabo *et al*., 2002). LAB have an ability to produce organic acid from carbohydrates through the process called fermentation. The fermentation system of LAB is divided into three groups based on fermentation of carbohydrate under unlimited condition; homofermentative, heterofermentative and facultative heterofermentative (Hutkins, 2006).

For millennia, people have used lactic acid fermentation, which is a natural process to preserve food and animal feed (Wood, 1985; Campbell-Platt and Cook, 1995). LAB transform sugar into lactic acid through the fermentation process under anaerobic conditions. Lactic acid is a natural, low-pH, effective and preservative compound. Sugar fermentation, followed by a reduction of the pH to a “safe” level of 4.5 or lower (Holzapfel, 1997) due to the production of lactic and other organic acids, is an important factor for the inhibition of growth

of undesired microorganisms. The low pH makes organic acids lipossoluble, allowing them to break through the cell membrane and reach the cytoplasm of pathogens by releasing lipopolysaccharides (Haller *et al*., 2001). Since both lactic and acetic acids are lipophilic, they can rapidly diffuse into the cell and dissociate at the near neutral pH of the cytoplasm releasing protons and anions until an equilibrium between the external and internal concentrations is reached. In this way cell growth can be inhibited if the accumulation of protons inside the cytoplasm exceeds the buffering capability of the cell or its capability to pump protons out by H^+-ATPase carriers (Corsetti *et al*., 2015).

1.8.2 Hydrogen peroxide (H_2O_2)

LAB have a capability to produce hydrogen peroxide in the presence of oxygen during the action of some enzymes such as Nicotinamide adenine hydroxyl dinucleotide oxidase (NAHD), Flavo-protein oxidase and glycerophosphate oxidase (Ross *et al*., 2002). LAB will not produce catalase for the removal of hydrogen peroxide (Ouwehand and Vesterlund, 2004). Collins and Aramaki (1980) reported that *Lactobacillus bulgaricus* has the ability to produce sufficient quantities of hydrogen peroxide at low temperature to reduce growth of psychotropic bacteria e.g. *Pseudomonas fragi*. Tharrington and Sorrells (1992) reported that the hydrogen peroxide produced by *Lactobacillus delbrueckii* subsp. *lactis* inhibited the growth of *L. monocytogenes*. Ito *et al*. (2003) showed that the cell-free filtrate of *Lc. lactis* subsp. *lactis* AI 62 containing 300-380 ppm hydrogen peroxide had a strong bactericidal effect against psychrotrophic food-borne pathogens such as *Listeria*, *Yersinia*, and *Aeromonas* species, and

mesophiles such as *E. coli.* Hydrogen peroxide is well studied and the mode of action by which it is one of the most powerful oxidizers is known. The destructive oxygen in hydrogen peroxide is known as a free radical. The peroxide is composed of two oxygen atoms, which react with bacterial cell walls' structures. The antimicrobial activity of hydrogen peroxide is due to the strong oxidizing effect on the bacterial cells, and to the destruction of basic molecular structures of cell proteins (Magnusson, 2003).

1.8.3 Carbon Dioxide (CO_2)

CO_2 is produced by heterofermentative LAB. The quantity of CO_2 is produced by LAB will change from one LAB to the other (Strom, 2005). Synthesis of CO_2 and the antimicrobial effect occur in anaerobic conditions (Cleveland *et al*., 2001). The antimicrobial activity of this compound is due to its ability to replace the existent molecular oxygen in food products, creating anaerobic conditions toxic to some aerobic food microorganisms such as moulds and Gram-negative bacteria through its action on the membranes of microbial cells and its ability to reduce internal and external pH (Caplice and Fitzgerald, 1999). Moreover, the antimicrobial activity of CO_2 is due to the inhibition of enzymatic de-carboxylation and accumulation of CO_2 in the membrane lipid bi-layer resulting in defective function in membrane permeability (Lindgren and Dobrogosz, 1990).

1.8.4 Diacetyl

Diacetyl is an aroma compound (2,3-butanedione) that is produced by a lot of LAB as a result of pyruvate and citrate metabolism. Diacetyl has a buttery odour.

It is found in most fermented products, especially dairy products and products from lactic acid fermentation. Diacetyl show more inhibitor effect on Gram-negative bacteria, fungi and yeasts than Gram-positive bacteria. Also LAB are highly resistant to diacetyl (Jay, 1982; Cogan and Hill, 1993; De Vuyst and Vandamme, 1994; Magnusson, 2003). Helander *et al*. (1997) reported that diacetyl can enter the cell of a Gram-negative bacteria throughout the porin protein, without altering the outer membrane permeability. The proposed mode for antimicrobial action relies on the reaction between diacetyl and the periplasmic proteins binding the arginine, thus interfering with the metabolism of such an amino acid. The Lack of similar binding proteins and the possibility to use a wider pattern of amino acids confer the Gram-positive bacteria a greater resistance to this compound (Jay *et al*., 2005). Diacetyl concentrations can be present in fermented foods in a range from 0.5-20 to 44.0-66.5 mg/kg in dairy and bakery products, respectively, as reported by Escamilla *et al*. (2000). However, diacetyl may act synergistically with other antimicrobial factors and contribute to combined preservation systems in fermented foods (Jay, 1982).

1.8.5 Bacteriocins

Bacteriocins of LAB are ribosomally synthesised peptides or proteins that have bactericidal or bacteriostatic activity (De Vuyst and Vandamme, 1994; Chen and Hoover, 2006). In recent years, LAB bacteriocins have generated interest due to their potential as a safe bio-preservative. LAB bacteriocins play an important role in food industries to improve quality and increase safety and shelf life of food products (De Vuyst and Leroy, 2007). LAB bacteriocins are different if compared with antibiotics as follows (Table 1.3). Richard *et al*. (2006) reported

the possibility of using bacteriocins as alternatives to antibiotics to play an important role in preserving food and also to be used for medical treatment cases. Another important factor to consider will be the economic aspects or the cost of using bacteriocin in foods. One way to reduce the cost is to determine the optimum parameters for the production of a bacteriocin. For economical use in food, the bacteriocins have to be produced in large amounts, preferably by growing the strains in media containing food grade ingredients (Ennahar *et al*., 2000). LAB and their bacteriocins have an inhibitory spectrum against microorganisms which includes food spoilage and pathogenic microorganisms (Schillinger *et al*., 1996).

Table 1.3: The comparison between bacteriocins and antibiotics which is explained by Cleveland *et al*. (2001)

Characteristics	**Bacteriocins**	**Antibiotics**
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Producer immunity	Yes	No
Target cell resistance or tolerance	Usually adaptation affecting cell membrane	Usually a genetically transferable determinant
Interaction requirements	Sometimes docking	Specific target molecules
Mode of action	Mostly pore formation	Cell wall or intracellular targets
Toxicity / Side effects	None known	Yes

LAB bacteriocins have several important traits that change them to an appropriate substance to be used as a food preservative such as: protein nature, low molecular weight, non-toxicity to laboratory-tested animals' food and a wide lethal activity against Gram-positive and Gram-negative bacteria, which include various pathogens bacteria such as *Listeria monocytogenes*, *Bacillus cereus*,

Salmonella sp., and *Staphylococcus aureus*. Recently, bacteriocins have become of great interest to food manufacturers who utilize them as bio-preservatives in food products in general and in perishable food in particular (Parada *et al*., 2007).

1.8.5.1 Classification of bacteriocins

Klaenhammer (1993) classified bacteriocins produced by LAB into four major classes. Class I: Lantibiotics that have a molecular weight of less than 5000 Dalton and contain to unnatural amino acids like Lanthionine, e.g. nisin which is produced by *Lactococcus lactis* subsp. *lactis*. Class II: Small heat-stable bacteriocins are bioactive peptides which do not contain Lanthionine and have a molecular weight of less than 10000 Dalton. Class III: Large heat-labile bacteriocins have a large molecular weight; there is meagre information available on this group of bacteriocins. Class IV: Complex proteins that require additional carbohydrate or lipid to attain antimicrobial activity. The majority of the bacteriocins that are produced by LAB associated with food belong to classes I and II (Lowe and Arendt, 2012).

1.8.5.2 Structure of nisin and mode of action

Nisin is a protein compound produced by *Lactococcus lactis* subsp. *lactis* with antimicrobial activity (Delves-Broughton *et al*., 1996; Kuwano *et al*., 2005). It is the only bacteriocin which is used for preserving food products such as cheese, beer and canned foods in more than 50 countries, and it has generally been

considered nontoxic (Harris *et al*., 1992; Delves-Broughton *et al*., 1996; Lacroix, 2010). Nisin is a bacteriocin which consists of 34 amino acids and is heat and acid stable. The molecular weight of nisin is 3500 Dalton (Roberts *et al*., 1992; Benech *et al*., 2002). Nisin has an antimicrobial activity against Gram-positive bacteria and the spore forming of bacteria, and it has an effect on the outer membranes of Gram-negative bacteria (Delves-Broughton, 1990; Gandhi and Chikindas, 2007; Settanni and Corsetti, 2008). Additionally, nisin has been used as a biopreservative and a potential agent in other fields such as in pharmaceutical, veterinary and health care products (De Arauz *et al*., 2009). The mechanism of nisin is very specific to the destruction of Gram-positive bacteria. Nisin attaches to a cell membrane lipid as shown (Figure 1.2) then inserts itself into the cytoplasm membrane by forming pores, which results in inhibition or death of the bacterium (Brötz and Sahl, 2000; Delves-Broughton, 2005).

There are three nisin variants were discovered which are nisin A, nisin Z, and nisin Q and they differ only in a few amino acids (Figure 1.3) (Twomey *et al*., 2002; Zendo *et al*., 2003). Today, nisin is the only bacteriocin available commercially which is manufactured industrially from supernatant of *Lc. lactis* subsp. *lactis* culture by a low-cost and simple process (Waites *et al*., 2001). In general, nisin Z is used more than nisin A as a preservative in food products because nisin Z has a higher inhibitory activity and solubility (Benech *et al*., 2002).

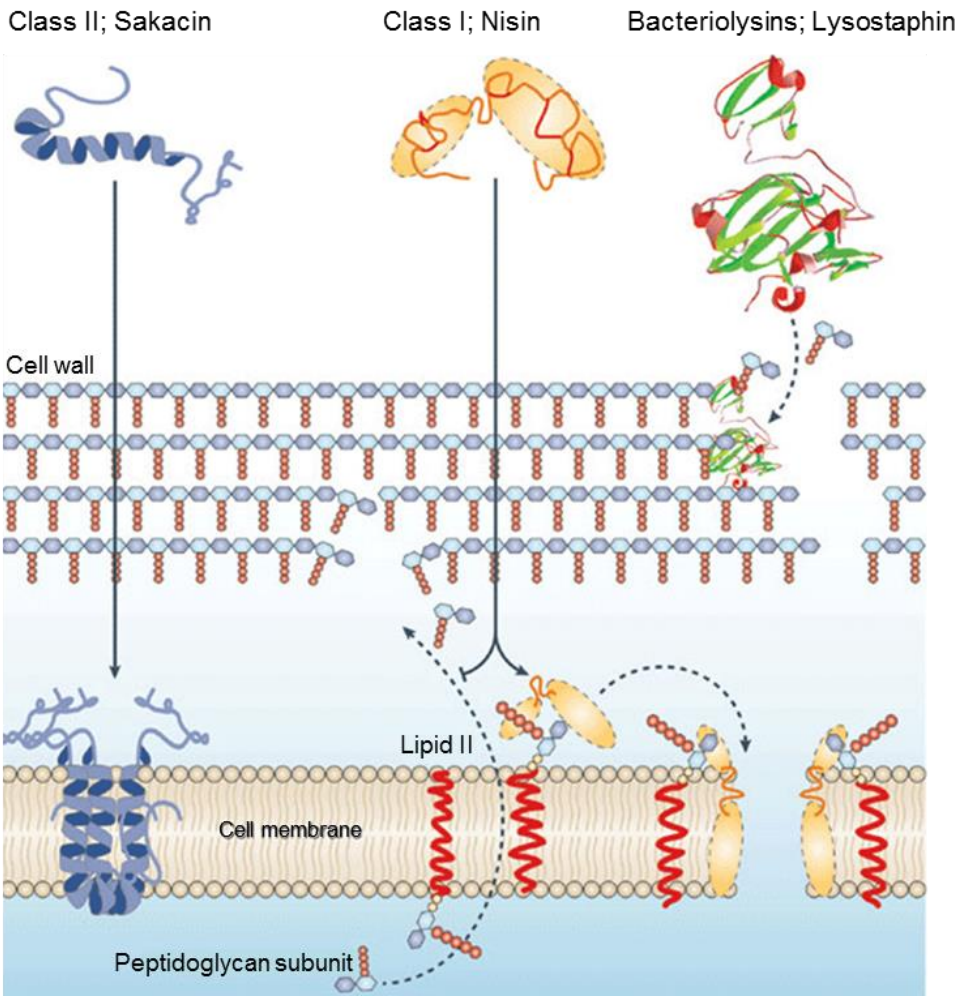


Figure 1.2: Mode of action of bacteriocins by LAB (Juodeikiene *et al*., 2012)

Nisin inhibits the majority of the Gram-positive bacteria tested so far and also many Gram-negative bacteria if presensitised with chelating agents such as ethylene diamine tetra acetate acid (EDTA), osmotic shock, sublethal heat, or freezing, to convert the cytoplasmic membrane accessible to nisin (Delves-Broughton, 1993; Blackburn *et al*., 1997; Gänzle *et al*., 1999).

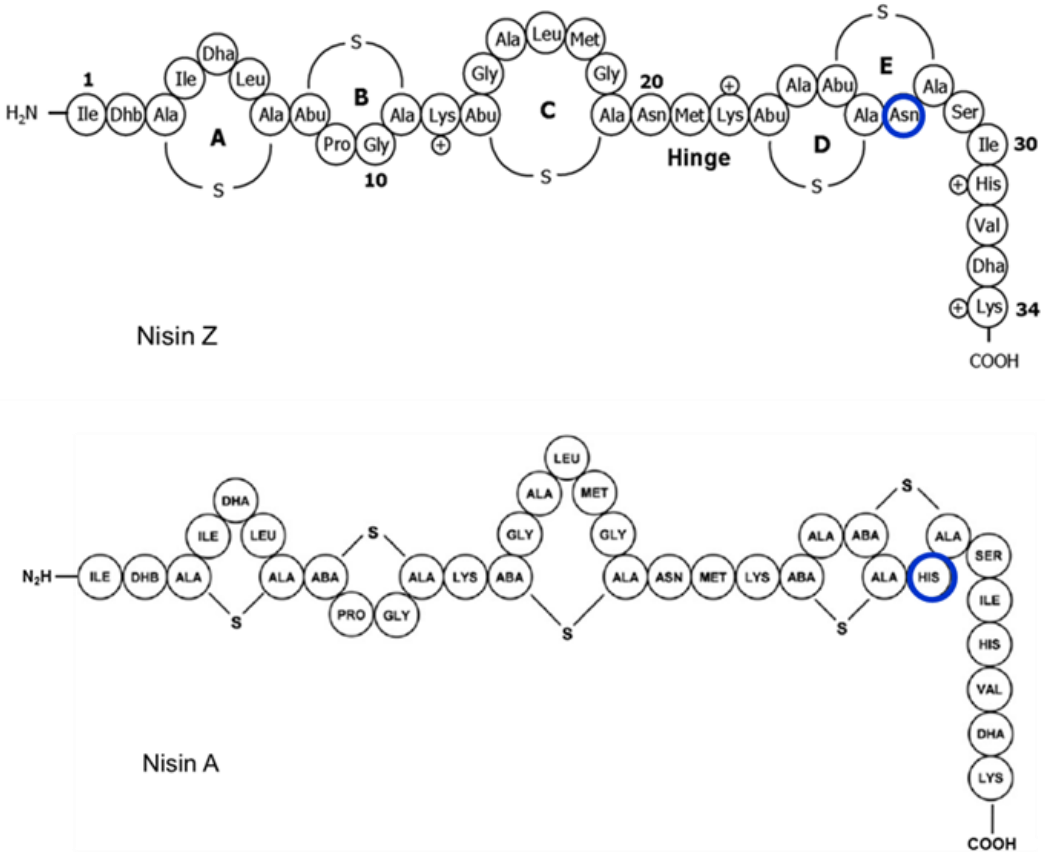


Figure 1.3: Structure of nisin A and nisin Z (Gross and Morell, 1971; De Vuyst and Vandamme, 1994)

Both vegetative cells and spores of bacilli were reported to be sensitive to nisin (Ray, 1992; DeVuyst and Vandamme, 1994). Spores of a sensitive strain were claimed to be more sensitive to nisin than the vegetative cells (Delves-Broughton *et al*., 1996). Nisin possibly inhibits the spores during the early stages of germination (Ray, 1992).

The inhibitory effect of nisin alone against different *Bacillus* sp. has been shown in laboratory media and in foods, as well as in combination with physical factors e.g. heat, pulsed-electric field and high hydrostatic pressure and chemical factors e.g. acids, monolaurin, sucrose fatty acid esters, and carvacrol (Ray,

1992; Roberts and Hoover, 1996; Thomas *et al*., 1998; Beard *et al*., 1999; Kato *et al*., 1999; Komitopoulou *et al*., 1999; Pol and Smid, 1999; Pol *et al*., 2000).

Jenson *et al*. (1994) quoted Lee (1988) who in a New Zealand conference reported a number of local food poisoning outbreaks due to the growth of *Bacillus cereus* in crumpets with no more information available. Bennett *et al*. (2013) reported that there was no outbreaks caused by *Bacillus cereus* in baked goods in United States from 1998-2008. While, the BIOHAZ Panel of EFSA 2016 reported 8 number of foodborne outbreaks from 67 human cases resulting in 2 hospitalisations from 2008-2012 caused by *Bacillus cereus* in bakery products. Rosenquist *et al*. (2005) reported that 40 number of *Bacillus cereus* group strains isolated in 53 samples of ready-to-eat-bread in Denmark which 31 out of the 40 isolates were classified as *Bacillus thuringiensis*. Emetic toxin was related to only one *Bacillus cereus* strain, while others involved in human diarrhoeal disease. Nisin is permitted as a preservative to control the growth of *Bacillus cereus* in crumpets. Addition of nisin to the batter mix at 3.75 mg/kg to prevent the growth of *Bacillus cereus* spore has received regulatory approval in Australia and New Zealand (Jenson *et al*., 1994).

Nisin is water soluble and stable at pH≤4, also at high temperatures (Ray, 1992). Its antimicrobial activity is best at low pH and it becomes inactive at pH 8 (Ray, 1992). Nisin can be inactivated by proteolytic enzymes such as α-chymotrypsin, pancreatin, and ficin that are able to break its peptidic chain then destroy its antibacterial properties (Ray, 1992). However, other enzymes such as trypsin, pepsin and carboxypeptidase have no significant effect on its antimicrobial effect (Chollet *et al*., 2008). One explanation for the insensitivity of some *Bacillus* sp. to nisin may thus be the production of proteolytic extracellular

enzymes. Because of the sensitivity of nisin to proteolytic enzymes, It is likely that nisin does not persist after being discharged into the environment.

Oshima *et al*. (2014) reported that nisin A can extend the shelf life of high-fat chilled dairy dessert (milk-based pudding) to 20 days by controlling the growth of spores from *Bacillus thuringiensis*, *Bacillus cereus* and *Paenibacillus jamilae*, when using reduced heat treatment to improve the flavour and aroma. Behnam *et al*. (2015) found that treatment of vacuum packaged rainbow trout (fish) with nisin resulted in improvement of quality and extension of the shelf life from 12 to 16 days at 4°C.

1.8.5.3 Usage of natural and commercial additives of nisin: (Nisin and its applications)

Nisin was first introduced commercially as a food preservative in the UK approximately 50 years ago (Delves-Broughton *et al*., 1996). Nisin A is currently the only bacteriocin licensed as a food preservative by FSA in the current EU where it is numbered (E234). Nisin generally regarded as a safe (GRAS) status by the US Food and Drug Administration (FDA) and World Health Organization (WHO) since 1969. Nisin products are mainly commercialised by DuPont Danisco (Nisaplin® and Novasin™) as dried concentrates containing approximately 2.5% nisin (Lacroix, 2010). Nisin is a naturally produced compound with antimicrobial activity. Applications for nisin have been described for food, feed and cosmetic preservation (Taylor, 1986), and disinfecting of surfaces (Daeschel and Mcguire, 1995). The potential applications of nisin were first suggested in 1951, which showed that the use of nisin-producing starter

cultures could prevent clostridial gas formation in cheese (Delves-Broughton *et al*., 1990). The major food uses are processed cheese products, other milk products and canned foods (Ray, 1992). Maximum level of addition is regulated in some countries, usually to 12-200 mg/kg (Ray, 1992). Levels of nisin are covered by Regulation (EC) No. 1129/2011 of the UK and EU and approved for use in foods which the maximum level is 12.5mg/kg or mg/l as appropriate. The level of natural and commercial additive nisin and the commercial extract nisaplin are mentioned as follows (Table 1.4). The dosage of nisaplin is in the range 25-500 (mg/kg, mg/L) in food as recommended by DuPont Danisco Company (DuPont Danisco, USA).

Nisin has been used as an additive to be effective in controlling spoilage and pathogenic bacteria in many food products (Jenson *et al*., 1994; Cooksey, 2005; Sivarooban *et al*., 2007). However, the activity of nisin may be affected by many factors, such as concentration, the target microorganisms, interaction with food components, fat content and phosphate type, processing and storage conditions of food (Davies *et al*., 1999; Soriano *et al*., 2004; Chollet *et al*., 2008).

Table 1.4: Levels of natural and commercial additive nisin and the commercial extract nisaplin in food application (Thomas *et al*., 2000; Delves-Broughton, 2005)

Food application	**Level of nisin (mg/kg, mg/L)**	**Level of Nisaplin (mg/kg, mg/L)**	**Typical Target Organism**
Processed cheese	2.5 – 15.0	100 – 600	*Bacillus, Clostridium*
Milk and milk products	0.25 – 1.25	10 – 50	*Bacillus (B. sporothermodurans)*
Pasteurised chilled dairy desserts	1.88 – 5.00	75 – 200	*Bacillus, Clostridium*
Liquid egg	1.25 – 5.00	5 – 200	*Bacillus (B. cereus)*
Pasteurised soups	2.50 – 6.25	100–250	*Bacillus* sp.
Crumpets	3.75 – 6.25	150 – 250	*B. cereus*
Fruit juice (pasteurised / ambient storage)	0.75 – 1.50	30 – 60	*Alicyclobacillus acidoterrestris*
Canned food (high acid)	2.5 – 5.0	100 – 200	*B. stearothermophilus, Clostridium botulinum, Cl. thermosaccharolyticum*
Dressings and sauces	1.25 – 5.00	50 – 200	Lactic acid bacteria, *Clostridium* sp., *Bacillus* sp.
Meat products such as bologna, frankfurter sausages	5 – 10	200 – 400	Lactic acid bacteria *Brochothrix thermosphacta Listeria monocytogenes*
Ricotta cheese, Beer, wine, fermented beverages, spirits	2.5 – 5.0	100 – 200	*Listeria monocytogenes* Lactic acid bacteria (*Lactobacillus*, *Pediococcus*)
Pitching yeast wash	25.0 – 37.5	1000 – 1500	
Reduced pasteurisation	0.25 – 1.25	10 – 50	
During fermentation	0.63 – 2.50	25 – 100	
Post fermentation	0.25 – 1.25	10 – 50	

1.8.6 Production of exopolysaccharides (EPSs)

A number of LAB are capable of producing exopolysaccharides (EPSs) which are excreted outside the cell in the form of capsules (Laws *et al*., 2001). This is a ropy material and sticks to the cell wall. This may be able to protect the microbial cell against desiccation, phagocytosis and phage attack (Cerning, 1990). The synthesis of EPSs by LAB has gained increasing interest for its ability to improve textural properties of fermented dairy products and non-dairy products such as cheese, buttermilk and fermented milks (Crescenzi, 1995; Ruas-Madiedo *et al*., 2002; Galle *et al*., 2012). Also, LAB play an important role in the development of the dough structure through the production of EPS that positively influence the viscosity of sourdough (Vogel *et al*., 2002). EPS in food products leads to increased density, balance, emulsion gel cofactor and water retention capacity (Crescenzi, 1995). Commercially, only a few bacterial EPSs used, such as xanthan, gellan, and dextran, due to high production costs (Freitas *et al*., 2011).

Li *et al*. (2014) reported that EPS produced from *Bifidobacterium bifidum* WBIN03 and *Lactobacillus plantarum* R315 exhibited antimicrobial activities against tested pathogens such as *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Staphyloccocus aureus*, *Cronobacter sakazakii*, *Candida albicans*, *Salmonella typhimurium*, and *Shigella sonnei* at 300 µg/mL using agar diffusion assay. A few studies showed that EPS from microorganisms had strong antimicrobial activity against several pathogens *in vitro*. Moreover, they have proposed several antibacterial mechanisms of EPS such as impairing cell division, disrupting the cell wall and cytoplasmic membrane, and decomposing DNA (He *et al*., 2010; Wu *et al*., 2010). Further studies are needed to evaluate

the components responsible for antimicrobial activity and mode of actions of EPS.

Cerning (1995) pointed out that the level of EPSs production from LAB depends on the growth stage, temperature, pH value, carbon sources from carbohydrates, and factors that can affect the growth indirectly. Usually milk (non-fat skim milk) is used as a medium to produce EPSs from LAB and some mucus and ropy materials appear as indicators for EPSs production (Mozzi *et al*., 2001). Ricciardi and Clementi (2000) and Ruas-Madiedo *et al*. (2002) found that several species of *Lactobacillus* can excrete EPSs. Vaningelgem *et al*. (2004) reported that supplementation of the skim milk medium with whey protein concentrate or whey protein hydrolysates increased EPS production by *Streptococcus thermophilus* ST111. The use of whey protein concentrate increased buffering capacity of the medium, thus decreasing the acidic effects on EPS production during fermentation (De Vuyst and Degeest, 1999).

The EPSs produced from LAB are classified into two groups: homo-polysaccharides, which are comprised of one type of monosaccharides, and hetero-polysaccharides which are comprised of repeated units of polysaccharide and non-carbohydrates units, including phosphate, acetyl and glycerol (Ruas-Madiedo *et al*., 2002). Homo-polysaccharides are secreted outside the cells through secreting enzymes such as glycansucrases which have a group of monosaccharides (Duboc and Mollet, 2001; Jolly *et al*., 2002). The hetero-polysaccharides are classified into two groups: fructan, which includes lefan and inulin, and the other group, glucan, which includes dextran, mutan, alteran beta 1-3 glucan (Monsan *et al*., 2001). The hetero-polysaccharides are produced inside the cell. A plasma membrane uses

nucleotides sugars to produce a series of polysaccharides (Cerning, 1995). It has been found that a number of LAB species are able to produce hetero-polysaccharides (Van den Berg *et al*., 1995). This type of sugar usually includes glucose, galactose, ramenose and small group of N-acetylglucosamine, N-acetylgalactosamine and glucoronic (Ruas-Madiedo *et al*., 2002).

1.9 Safety concerns and shelf life of bakery products

Bread is a food product that is universally accepted as a very convenient form of food over the world. It has desirability to all sectors of the population rich and poor, rural and urban, which gives cereals an important position in international nutrition (Potter and Hotchkiss, 2006). It is a good source of nutrients, such as macro-nutrients e.g. high starch content as an energy source, protein and lipids rich in essential fatty acids, and micro-nutrients e.g. minerals (Calcium, iron, magnesium and zinc), vitamins, especially many B vitamins and vitamin E, antioxidants and phytochemicals that are all essential for human health (Potter and Hotchkiss, 2006; Dewettinck *et al*., 2008). Bread has been transformed into different types with varying characteristics depending on the innovations put into the production. All these varying attributes of bread most times distract consumers from the nutritional and wholesome quality of the bread product. This is to say that there is a need to continuously, improve the nutritional and organoleptic attributes of bread (Potter and Hotchkiss, 2006).

Bread is one of the most important products of wheat flour in many parts of the world, especially in developing countries. The extension of shelf life by decreasing the growth of pathogenic microorganisms and delaying bread staling

during storage period is one of the biggest challenges for the baking industry today (Plessas *et al*., 2008).

Bakery products are classified in three groups based on pH such as high acid with pH <4.6, low acid with pH>4.6 but <7 and non-acid products with pH>7 (Smith *et al*., 2004). According to another classification based on their Aw, the categories are as follows; low moisture bakery products (Aw<0.6), intermediate moisture bakery products (Aw between 0.6 and 0.85) and high moisture bakery products (Aw>0.85 and generally between 0.95 and 0.99) (Smith and Simpson, 1995). In high moisture products (aw 0.94–0.99), almost all bacteria, yeasts, and moulds are capable of growth (Smith, 1992). Several types of bakery products can be found on market shelves in categories shown in Table 1.5. Bakery products are subjected to chemical, physical and microbial spoilage, influenced by interrelated factors. These factors are storage temperature, relative humidity, preservatives, pH, packaging material and gas around product, and quite importantly the moisture content and Aw (Smith *et al.*, 2004).

Examples of physical spoilage are staling, which reduces consumer acceptability, and moisture loss and gain, which is a serious problem as it results in textural changes and may promote chemical and microbiological spoilage in low and intermediate moisture bakery products (Hebeda and Zobel, 1996; Smith *et al*., 2004).

Chemical hazards occur when chemicals are present in foods at levels that can be harmful to humans. In the food industry, there are dissimilar kinds of chemical hazards, e.g. mycotoxins, pesticides, environmental contaminants (air and water) and food additives which may result in food contamination.

Mycotoxins are secondary metabolites made by filamentous fungi. These mycotoxins may result in a toxic response, known as mycotoxicosis, when consumed by humans or animals. *Aspergillus*, *Fusarium*, and *Penicillium* are the most predominant mycotoxin producers, contaminating foods through fungal growth before and during harvest, or often during improper storage (Theron and Lues, 2011). A mycotoxin commonly related to bread products is ochratoxin A, which mainly is present in the wheat flour (Arroyo *et al*., 2005). Pesticides are used in cereals to prevent pest growth and storage problems, associated with a risk of residues in the cereal and cereal products if used inadequately (Berry, 2006).

High fat bakery goods are particularly vulnerable to rancidity, a chemical spoilage characterised by lipid degradation resulting in off-odours, off-flavours and changes in the colour during processing and storage, which render products unpalatable and decrease the shelf life. Microbial spoilage (yeast, mould, bacterial growth) is often the major factor limiting the shelf life of high and intermediate moisture bakery products, and is also a major cause of economic loss to the bakery industry. These microorganisms often spoil food by growing in the food and producing substances which change colour, texture and odour of the food, making it unfit (undesirable) for human consumption (Smith *et al*., 2004).

Table 1.5: Categories of bakery products found on supermarket shelves. Adapted from Seiler (2012)

Categories of bakery products	Types within each category
Unsweetened products	Bread: sliced, crusty, par-baked, ethnic Rolls: soft, crusty Crumpets, Sourdough bread, Naan bread, flat bread, Tortilla, English muffins, Pitta bread, Croissants, Pizza base, Raw pastry
Sweet products	Large cakes: plain, fruited Pancakes, Doughnuts, Waffles Cookies, Biscuits, American muffins Buns, Wafers
Filled products	Tarts: fruit, jam Pies: meat, fruit Sausage rolls Pasties, Pizza, Quiche Cakes: cream, custard, filled pastries

A number of methods are applied to reduce microbial spoilage in bread products such as addition of propionic acid, modified atmosphere packaging, irradiation and pasteurisation of packaged bread (Legan, 1993; Pateras, 1998). Nowadays, the increased interest of the scientific community in the application of consumer-friendly bio-preservatives is due to the increased concern of the consumers regarding the side effects of chemical preservatives, and also the growing demand for minimally processed foods with long shelf life (Corsetti *et al*., 2015).

LAB are used in many bakery products in different ways due to their antimicrobial activities and also for improving the quality, safety and overall

acceptability of the end product. Many published studies have been carried out on LAB microbiota in order to find effective antifungal strains, and most of them refer to *Lactobacillus* strains (Schnürer and Magnusson, 2005). Magan and Aldred (2006) claimed that colonisation and growth of fungi represents more than 90% of the total microbial contamination which causes spoilage of wheat bread and other bakery products. Rizzello *et al*. (2011) reported that various fungi isolated from bakery products were inhibited by *Lb. plantarum* and *Lb. rossiae* isolated from raw wheat germ. During fermentation, organic acids and peptides synthesised *in-situ* were responsible for the antifungal activity. However, the inhibitory activities of the characterised components were different, depending on the choice of LAB strains and flour type (Rizzello *et al*., 2011). Dal Bello *et al*., (2007) characterised lactic acid, phenyllactic acid, cyclic dipeptides cyclo (L-Leu–L-Pro) and cyclo (L-Phe–L-Pro) produced by *Lb. plantarum* FST 1.7 and found them to inhibit the growth of *Fusarium* sp. in wheat bread. Lavermicocca *et al*. (2000) reported that strains isolated from sourdough showed strong antifungal activity by a conidial germination assay. A concentrated *Lb. plantarum* culture filtrate of almost completely inhibited *Eurotium repens*, *Eurotium rubrum*, *Penicillium corylophilum*, *P. roqueforti*, *P. expansum*, *Endomyces fibuliger*, *Aspergillus niger*, *A. flavus*, *Monilia sitophila* and *Fusarium graminearum*. Gerez *et al*. (2009) reported that four LAB strains isolated from sourdough (*Lactobacillus plantarum* CRL 778*, Lactobacillus reuteri* CRL 1100, *Lactobacillus brevis* CRL 772, and *L. brevis* CRL 796) could inhibit *Aspergillus*, *Fusarium*, and *Penicillium* which are the main contaminants in bread. Indeed, LAB and their bacteriocins have been used as an additive to prevent the growth of *Bacillus* species and spore forming bacteria in bread

(Gänzle, 1998). For example, nisin; produced by *Lc. lactis* subsp. *Lactis*, has been used as additive to the batter of crumpets at 3.75 mg/kg to prevent the growth of *Bacillus cereus* spore and to prolong shelf life (Jenson *et al.*, 1994).

1.10 Overview of sourdough technology

Sourdough technology is widely used in bread making and cake production as it confers distinctive characteristics such as palatability, high sensory properties and shelf life to the resulting products (Corsetti and Settanni, 2007; Banu *et al*., 2011; Gobbetti and Gänzle, 2013). Sourdough has become a component of recent biotechnology of pastry products, in particular bread production, as a leavening agent (Hansen, 2012). The sourdough is made from flour and water and then is fermented with LAB and yeast to produce a pH value around 4.5. The amount of LAB in sourdough is between 10^8-10^9 CFU/g, and the LAB: yeast ratio is generally 100:1 (Gobbetti *et al*., 1994; Ottogalli *et al*., 1996; Meignen *et al*., 2001; Stolz, 2003). The LAB that is added to sourdough is preliminarily comprised of heterofermentative strains, elaborating lactic acid and acetic acid in the mixture. These confer a sour taste to the end product (De Vyust and Neysens, 2005). Recently, sourdough is employed in the manufacture of breads, cakes and crackers (Ottogalli *et al*., 1996). The main role of LAB (mainly obligatory and facultative heterofermentative lactobacilli) is in the acidification process, while yeasts mainly account for the leavening of the dough by releasing CO_2 (Hammes and Gänzle, 1998). Sourdough bread properties are pH (pH 3.8-4.6), lactic acid (0.4-0.8%), acetic acid (0.1-0.4%),

and slow staling and it has a good protection against microbial contaminations (Hansen *et al*., 1989).

LAB ensure acid production and leavening upon addition of flour and water. The fermentation of sourdough is a sophisticated process caused by the effects of the metabolism of both yeasts and LAB. In dough, sometimes yeast and LAB work synergistically. Despite its main role as fermenter of carbohydrate and producer of ethanol and carbon dioxide, the yeast also produces by-products which confer taste and flavour in the final-product (Faid *et al*., 1993; Meignen *et al*., 2001). Sourdoughs made with both LAB and yeasts resulted in more aroma compounds compared to sourdoughs made from single starters based either on LAB for example *Lb. brevis* or yeast such as *S. cerevisiae* (Meignen *et al*., 2001). Therefore, this increase in the production of aroma compounds in a mixed-starter process appears to be related to the proteolytic activity of LAB (Corsetti and Settanni, 2007). According to Spicher and Nierle (1988), LAB cause the release of amino acids which will then be used by *S. cerevisiae* to produce higher alcohols. A correlation would exist between concentrations of amino acids and aroma compounds but specific amino acids could not be associated with corresponding volatile compounds (Torner *et al*., 1992). Additionally, a higher percentage of sourdoughs could be produced with a combination of *S. cerevisiae* with *Lb. sanfranciscensis* and *Lb. plantarum* during the sourdough fermentation process (Gobbetti *et al*., 1995a).

The purpose of using sourdough in bread production involves: using microbes as leavening agent with little or no baker's yeasts, improving of dough characteristics, conferring flavour and taste to the end-product, upgrading the

product's nutritional value and prolonging shelf life of the final-product (Arendt *et al*., 2007; Dal Bello *et al*., 2007; Nawaz *et al*., 2007).

In bread production, organic acids or approximately 15% of sourdough can be added to common dough to avoid the growth of microbial contaminants such as moulds or bacteria (e.g. *Bacillus subtilis* and *clostridia*) that subsequently grow and spoil the product (Voysey and Hammond, 1993). The antimicrobial activity of sourdough arises from lactic acid, acetic acid, carbon dioxide, diacetyl, ethanol, hydrogen peroxide and bacteriocins produced by LAB during fermentation. Because of their effective antimicrobial role, bacteriocin-producing *Lactobacillus* strains are commonly used in sourdough (Voysey and Hammond, 1993; Rosenquist and Hansen, 1998; Vogel *et al*., 1999). Additionally, it can improve dough and bread quality and prolong shelf life of the final product. Moreover, sourdough fermentation is a safe and acceptable technique to protect bread from detrimental effects by microbial agents and it could meet the demand of the consumers for safe and natural additive free goods (Messens and De Vuyst, 2002; Corsetti, 2013).

The sourdough mechanism is sophisticated and there are several parameters which can influence the metabolic efficiency of the microflora production of sourdough, such as flour type and processing factors (Hammes and Gänzle, 1998). Temperature can also play an important role as it could change the metabolism and fermentability environment for LAB and yeast. During fermentation, biochemical alterations take place in the flours biopolymers such as protein and carbohydrate as a result of microorganisms and its indigenous fermentative activity (Hammes and Gänzle, 1998).

1.10.1 LAB in sourdough fermentation and their Influence on sourdough

There are numerous species of LAB which have been isolated from sourdough. It has been mentioned that a number of LAB species occur in wheat and rye flour such as strains of *Lactobacillus, Pediococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Weisella* (Hammes and Vogel, 1995; De Vuyst and Neysens, 2005; Ehrmann and Vogel, 2005). About 50 different species of LAB from sourdough have been reported by De Vuyst and Neysens (2005) and Hammes *et al*. (2005). The majority of LAB of the genus *Lactobacillus* has been isolated from sourdoughs (Ottogalli *et al*., 1996; Corsetti *et al*., 2001; Corsetti and Settanni, 2007) such as *Lactobacillus sanfranciscensis*, *Lb. brevis* and *Lb. plantarum* (Vogel and Hammes, 1990; Gobbetti, 1998; Corsetti *et al*., 2001; Corsetti *et al*., 2003). The population dynamics of microbial food ecosystems have been studied mainly through microbiological analysis (Giraffa, 2004). In recent years, culture-independent methods have been developed to circumvent the limitations of conventional cultivation for the analysis of microbial communities in fermented foods (Ercolini, 2004). In this regard, denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments (16S rRNA PCR-DGGE) is often used as a relatively rapid and reliable cultivation-independent method to study the biodiversity and population dynamics of microbial communities in fermented foods (Ercolini, 2004; Van Der Meulen *et al*., 2007). The application of PCR-DGGE technique has been described to monitor the diversity and dynamics of LAB and yeast populations during sourdough fermentation processes (Meroth *et al*., 2003b; Gatto and Torriani, 2004; Meroth *et al*., 2004; Randazzo *et al*., 2005). The PCR-DGGE technique is a genetic fingerprinting technique that examines the microbial

diversity based upon electrophoresis of PCR-amplified 16S rDNA fragments with gels containing a linear gradient of DNA denaturants (Muyzer *et al*., 1993). The PCR product banding pattern is indicative of the number of bacterial species or assemblages of groupings consisting of species that are present and thus allow visualisation of the genetic diversity of microbial populations (Muyzer *et al*., 1993). Many researchers still report on the existence of non-identifiable and perhaps new sourdough LAB species and/or strains (Rosenquist and Hansen, 2000; De Vuyst *et al*., 2002). When back-slopping is applied for sourdough fermentation, one can find the microflora of spontaneous sourdough fermentations (where homofermentative lactobacilli dominate), but mainly heterofermentative lactobacilli are found. The so-called sourdough lactobacilli *Lactobacillus sanfranciscensis* (Kline and Sugihara, 1971), *Lb. pontis* (Vogel *et al*., 1994), *Lb. panis* (Wiese *et al*., 1996), *Lb. paralimentarius* (Cai *et al*., 1999), *Lb. frumenti* (Müller *et al*., 2001), and *Lb. mindensis* (Ehrmann *et al*., 2003) are considered typical to sourdough environments.

The homo-fermentative species may not produce CO_2, but their main role is to acidify and improve flavour. Despite the fact that homo-fermentative species of LAB are used in the vast majority of the food products, hetero-fermentative species also play a key role in the fermentation of sourdough (Corsetti *et al*., 2001; Corsetti *et al*., 2003). This is because only heterofermentative LAB are able to produce a significant amount of acetic acid under anaerobic conditions which is of interest in sourdough to improve taste and flavour of the bread (Kosmina, 1977). In contrast, homofermentative LAB produce a considerable amount of lactic acid compared to acetic acid, hence it gives a flat taste and flavour to the bread (Spicher *et al*., 1981). In a study to compare the effect of

homo- and hetero- fermentative LAB on the aroma of rye-bread, it was found that using a pure culture of hetero-fermentative bacteria *L. brevis* resulted in the desirable flavour and taste and an elastic crumb; however, adding homo-fermentative bacteria (*Lb. plantarum)* to the bread led to undesirable flavour and taste. It has been suggested that both types should be added to obtain acceptable aroma and crumb properties (Oura *et al*., 1982).

Cereal fermentation by combination of both leavening agents (LAB and yeast), commonly referred to as sourdough or yeasted preferment, is a traditional and natural method, through metabolism of carbohydrates and of nitrogen sources, for improving the flavour, dough structure, texture and shelf life of the leavened baked good (Clarke and Arendt, 2005; Arendt *et al*., 2007). Gül *et al*. (2005) found that a mixture of *S. cerevisiae* (1.5%) and *Lactobacillus amylophilus* (1.5%) could produce sourdough and bread with the best quality attributes including yield and specific volume. However, a sensory evaluation revealed that consumers preferred sourdough bread made from a mixture of 1.5% *S. cerevisiae* and 1.5% *Lactobacillus sake* (Gül *et al*., 2005)

LAB during fermentation of sourdough may contribute to the improvement the quality of cereal products in dissimilar ways as shown in Figure 1.4 (Rollán *et al*., 2010). Through fermentation process in sourdough, LAB produce organic acids from carbohydrates which results in a decrease of pH value, inhibition of mould growth and microbial spoilage, prolonging shelf life of bread products (Rollán *et al*., 2005; Gobbetti *et al*., 2007; Gerez *et al*., 2008, Lhomme *et al*., 2016). However, they can produce flavour volatile compounds from gluten in the flour that can be affected by hydrolysing proline-rich allergenic fragments, improving the edibility and quality of whole grain, dietary fibre-rich products, gluten-free

products and increasing levels of bioactive compound and inducing mineral bioavailability (De Angelis *et al*., 2003; Arendt *et al*., 2007). This bioactive compound, such as the phenyllactic acid (derived from the phenylalanine metabolism), is active against several fungal species isolated from bakery products, flour and cereals, including bacterial contaminants (Lavermicocca *et al*., 2000; Gerez *et al*., 2009).

The hydrolysis of the flour protein during dough fermentation is of importance for bread quality. The sourdough fermentation causes increased amino acid concentrations, while in dough fermentation by yeast only, a decrease of these compounds has been detected (Gobbetti, 1998). Recent studies have explained the contributions of cereal and microbial enzymes to the proteolysis, peptide degradation and amino acid turnover during sourdough fermentation (Loponen *et al*., 2007; Rizzello *et al*., 2007). The proteolytic activity of LAB and active proteases of cereal flour under acidic conditions may be the cause of protein degradation. Microbial acidification of the dough can achieve the optimum pH (3.5-4) for the cereal proteinases to play an essential role in the primary proteolysis of gluten (Rollán *et al*., 2010). The released peptides are hydrolysed to amino acids (secondary proteolysis) by intracellular LAB peptidases, these metabolites may be further metabolised or accumulated in the dough (Figure 1.4).

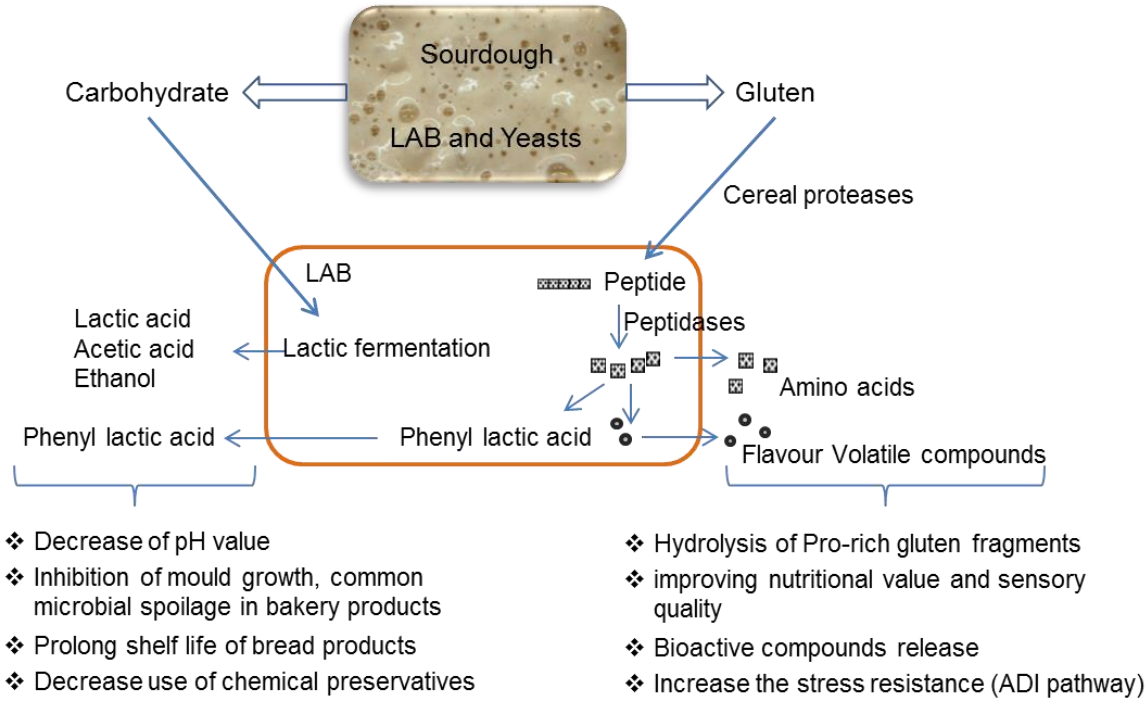


Figure 1.4: Role of LAB in sourdough fermentation. Adapted from Rollán *et al*. (2010)

1.10.2 Classification of sourdough

Sourdough is classified into three types on the basis of technological applications; type I, type II and type III (De Vuyst and Neysens, 2005; Corsetti and Settanni, 2007; De Vuyst and Vancanneyt, 2007; De Vuyst *et al*., 2009).

Type I sourdough is produced by a traditional method. In this type of sourdough, the process is characterised by continuous steps at fermentation temperature (22-28ºC) and pH 4, with the microorganisms kept metabolically active through daily refreshments. At present, this is the most widely used type which is used in the production of traditional breads.

Type I sourdoughs are known to have an effective role in achieving dough leavening without addition of baker's yeast. In this type of sourdough, a three-stage protocol is applied relying on three refreshments over 24h in order to obtain the leavened dough to bake. The steps are characterised by a given dough yield (DY), fermentation temperature and time. At the end of the last step of fermentation the sourdough is used as the leavening agent; so it can be counted as a natural starter culture having many microbial strains. The dominant strains of LAB in these sourdoughs are *Lb. sanfransciscensis*, *Lb. pontis*, *Lb. brevis*, *Lb. fermentum*.

Type II sourdough, or accelerated sourdough is utilised during bread making as source of souring. This is semi-solid and produced in a long-term fermentation (2-5 days). In this type of sourdough, fermentation temperature is increased to above 30ºC to speed up the process through increasing the activity of microorganisms which is generally have a shelf-life of several days (approximately one week in a cold environment). The dominant LAB in the type II sourdough are *Lb. pontis*, *Lb. reuteri*, *Lb. fermentum*, *Lb. brevis*, *Lb. delbrueckii* and *Lb. acidophilus* etc.

Type III sourdough is produced in a dry form and is characterised by having LAB resistant to drying processes. The LAB in these sourdoughs are resistant to drying and includes *Lb. plantarum* and *Lb. brevis* as shown in Table 1.6 (Hammes and Ganzle, 1998; Clarke *et al*., 2002). The quality of the sourdough bread is influenced by the microbial flora (starter cultures of LAB and yeasts), flour type (rye/wheat, flour extraction rate), flour/water ratio (dough yield), and the process parameters such as temperature, initial pH, quantity of sourdough incorporated in dough and time of fermentation (Müller *et al*., 2001).

The type II and III sourdoughs require the addition of baker's yeast (*Saccharomyces cerevisia*e) as a leavening agent, whereas type I does not require this addition (Müller *et al*., 2001; Corsetti, and Settanni, 2007). Sourdough LAB consisting of obligate and facultative hetero-fermentative and obligate homo-fermentative species are associated with type I, type II and type III sourdoughs. Type 0 dough, for which baker's yeast is the main fermenting agent, is not made with sourdough technology. Bacterial isolates from, a mature sourdough or other natural environment are selected and tested for their suitability for being employed as sourdough starters and their viability after drying. Lyophilized strains of *Lb. delbrueckii, Lb. fructivorans, Lb. plantarum,* and *Lb. brevis* have been established as sourdough LAB (Hammes and Gänzle, 1998). In contrast to the type-1 sourdough starters, frequent inoculation of these strains is required as these are not well adapted to the cereal environment (Röcken and Voysey, 1995). Due to the selective pressures that result from the environmental conditions of sourdough preparation, *Lb. sanfranciscensis* dominates type I sourdough fermentations (Corsetti *et al*., 2001; Foschino *et al*., 2001). The type Ib sourdough contains several LAB which refers to over 50 LAB species of *Lactobacillus*, *Pediococcus*, and *Leuconostoc* in concentrations of about 10^8–10^9 CFU/g (Gobbetti *et al*., 1994; Ottogalli *et al*., 1996; Stolz, 2003; De Vuyst and Neysens, 2005). Table 1.5 shows the Classification of sourdoughs and the corresponding characteristic microflora.

Table 1.6: Classification of sourdoughs and the corresponding characteristic microflora. Adapted from De Vuyst and Neysens (2005)

Types	Obligate heterofermentative	Facultative heterofermentative	Obligate homofermentative
Type Ia	*Lb. sanfranciscensis*		
Type Ib	*Lb. brevis* *Lb. buchneri* *Lb. fermentum* *Lb. fructivorans* *Lb. pontis* *Lb. reuteri* *Lb. sanfranciscensis* *W. cibaria*	*Lb. alimentarius* *Lb. casei* *Lb. paralimentarius* *Lb. plantarum* *Pc. pentosaceus*	*Lb. acidophilus* *Lb. delbrueckii* *Lb. farciminis* *Lb. mindensis*
Type Ic	*Lb. fermentum* *Lb. pontis* *Lb. reuteri*		*Lb. amylovorus*
Type II	*Lb. brevis* *Lb. buchneri* *Lb. fermentum* *Lb. fructivorans* *Lb. pontis* *Lb. reuteri* *Lb. sanfranciscensis* *W. confusa*		*Lb. acidophilus* *Lb. amylovorus* *Lb. delbrueckii* *Lb. farciminis* *Lb. johnsonii*
Type III	*Lb. brevis*	*Lb. plantarum* *Pc. pentosaceus*	

1.10.3 Use of sourdough in cereal products

The use of the sourdough process as a form of leavening is one of the oldest biotechnological processes in food production (Röcken and Voysey, 1995). The use of sourdough in wheat breads has gained popularity as a mean to improve the quality and flavour of wheat breads (Brummer and Lorenz, 1991; Corsetti *et al*., 2000; Thiele *et al*., 2002). To facilitate continuous production, one can save a portion of ripe sourdough to seed subsequent dough; this process has been

conducted since the nineteenth century (Williams and Pullen, 1998). A vast array of traditional products rely on the use of the sourdough fermentation to yield baked goods with a particular quality characteristic. Some examples include the well-known Italian products associated with *Christmas, Panettone*, which originated in Milan (Sugihara, 1977). San Francisco sourdough, French breads (Kline *et al*., 1970) and soda crackers (Sugihara, 1985) are other examples of wheat products that depend on the process of souring. The same process is also used in the production of a number of flat breads, a typical example of which is the Egyptian Baladi bread (Qarooni, 1996).

Anti-rope activity of *Bacillus* sp. has to be considered an important characteristic for selecting LAB in order to extend the shelf life of baked products (Rosenquist and Hansen, 1998; Pepe *et al*., 2003). For example, Pepe *et al*. (2003) reported that using *Lb. plantarum* E5 and *Lu. mesenteroides* A27 during the baking process had the most effective antirope activity and inhibited the development of ropiness for more than 15 days. In addition, the study also reported that the bread produced with *Lb. plantarum* E5 and *Lu. mesenteroides* A27 in a lower storage temperature (23°C) could extend the bread shelf life for 7 days, while in a storage temperature of 30°C it could be extended for 4 days.

Quintavalla and Parolari (1993) have described the importance of pH as a controlling factor in the development of ropiness. According to the study observations, the time of inhibition of rope symptoms increases at a low pH (range, pH 3.7 to 4.3). The differences may have been due to the different experimental conditions and the different strains used as indicators. The storage of bread at temperatures below 25°C is another factor associated with

the antirope activity of the starter in order to prevent the growth of *Bacillus* and prolong the shelf life of the bread (Quintavalla and Parolari, 1993).

1.10.4 Proteolysis and starch hydrolysis

Metabolic properties including production of lactic and acetic acids, synthesis of aroma substances, and proteolytic and amylolytic activities of sourdough LAB are of interest for their selection as sourdough starter cultures. During the fermentation of sourdough, proteolysis can be achieved by LAB through creating optimum conditions for activity of cereal proteinases. High proteolytic activity of LAB may cause the hydrolysis of wheat proteins in a strain-specific manner (Di Cagno *et al*., 2003). In general, sourdough fermented with LAB caused an increase of amino acid concentrations during fermentation. However, dough fermentation with yeast resulted in a decrease in the concentration of free amino acids. An intermediate value for total amino acid levels is produced by the combination of yeast and LAB. The factors that affect the level of individual amino acids in wheat doughs are the pH level of the dough, temperature, fermentation time and the consumption of amino acids by the fermentative microorganisms (Thiele *et al*., 2002).

Flavour components are key elements for consumer acceptance and product identification in bread. One category of speciality breads, the sourdoughs have a fermentation process affected by a complex microflora of yeasts and LAB which confer specific flavour characteristics (Rehman *et al*., 2006). LAB can produce important flavour components in sourdough fermentation which are presented in Table 1.7 (Damiani *et al*., 1996).

Table 1.7: Flavour components present in wheat flour sourdough fermented with hetero-fermentative and homo-fermentative LAB strains. Adapted from Damiani *et al*. (1996)

Compounds	homo-fermentative LAB					hetero-fermentative LAB				
	1	2	3	4	5	6	7	8	9	10
Lactic acid	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	+	-	-	-	+	+	+	+	+
Ehanol	-	-	-	-	-	+	+	+	+	+
1-Propanol	-	-	-	-	-	+	-	-	-	-
Ethyl acetate	+	+	+	+	+	+	+	+	+	+
Acetaldehyde	+	+	+	+	+	+	+	+	+	+
Hexanal	+	+	+	+	+	+	+	+	+	+
Octanal	+	+	+	+	+	+	+	+	+	+
Nonanal	+	+	+	+	+	+	+	+	+	+
Diacetyl	+	+	+	+	+	-	-	-	-	-
2-Methyl-1-pentanol	-	-	-	-	-	+	+	-	-	-
3-Methyl-1-butanal	+	+	-	-	-	+	-	-	-	-
Hexane	+	+	-	+	+	+	+	+	+	+
Heptane	+	+	+	+	+	+	+	+	+	-
Octane	+	+	+	+	+	+	+	+	+	-

+, Present and -, not present

1, *Lb. plantarum*; 2, *Lb. farciminis*; 3, *Lb. alimentarius*; 4, *Lb. acidophilus*; 5, *Lb. delbrueckii* 6, *Lb. brevis lindneri*; 7, *Lb. brevis*; 8, *Lb. fructivorans*; 9, *Lb. fermentum*; 10*, Lb. cellobiosus*

The typical sourdough flavours of baked breads are produced by bacterial proteolysis during sourdough fermentation if compared to the chemically acidified or yeasted breads (Hansen *et al*., 1989). The addition of amino acids such as ornithine, leucine and phenylalanine to doughs increased the flavouring compounds (Gassenmeier and Schieberle, 1995). The proteolytic strains of LAB may affect the level of amino acids in doughs, while cereal proteases can degrade proteins in sourdoughs (Thiele *et al*., 2003; Thiele *et al*., 2004).

The levels of aliphatic, dicarboxylic, and 22 hydroxyl amino acids in wheat sourdoughs is increased by *Lb. brevis* subsp. *lindneri*, *Lb. sanfranciscencis*, *Lb. brevis* and *Lb. plantarum* (Collar *et al*., 1991; Gobbetti *et al*., 1994). The microbial starter and the processing conditions affect proteolytic activity of wheat sourdough. The level of free amino acids relies on the extraction rate of flour and the fermentation temperature (Martínez-Anaya, 2003). It has also been reported that the level of amino acids and the amount of amino acids is affected by a dough yield (Martínez-Anaya, 2003).

It is well-known that amylolytic LAB found in different tropical amylaceous fermented foods have been prepared mainly from cassava and cereals (e.g., maize and sorghum). Strains of *Lb. plantarum* were isolated from African cassava-based fermented products (Nwankwo *et al*., 1989), and amylolytic LAB species *Lb. manihotivorans* has been isolated from cassava sour starch fermentations in Colombia (Morlon-Guyot *et al*., 1998). Agati *et al*. (1998) isolated amylolytic strains of *Lb. fermentum* for the first time in Benin maize sourdough (ogi and mawè). Amylolytic strains of *Lb. plantarum* and *Lb. fermentum* strains have been found in various Nigerian traditional amylaceous fermented foods by Sanni *et al*. (2002). The search for amylolytic LAB in fermented amylaceous foods has been justified by the high starch content of the raw material.

Lactic acid can be produced from the carbohydrate materials by coupling the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived glucose into a single step. This process has been utilised for lactic acid production from raw starch materials and many representative bacteria including *Lactobacillus* and *Lactococcus* species (Vishnu *et al*., 2002; Naveena

et al., 2003; Naveena *et al*., 2005). Several amylolytic LAB can convert the starch directly into lactic acid (Agati *et al*., 1998; Guyot and Morlon-Guyot, 2001; Santoyo *et al*., 2003; Thomsen *et al*., 2007). Sourdough bread is more easily digestible due to the macromolecule-extended enzymatic hydrolysis action by the LAB that facilitates hydrolysis (Katina *et al*., 2005).

1.10.5 Effect of ingredients and processing on the sourdough flavour

The LAB strains of sourdough vary in metabolism and aroma compounds. Monoculture fermentation of dough for 15h at 30ºC, followed by mixing and further 10h fermentation has shown increase in the production of sourdough volatile compounds (Meroth *et al*., 2004). Using only yeasts in wheat bread, seven volatiles were found to be abundant: acetaldehyde, acetone, ethyl acetate, ethanol, hexanal, isobutyl alcohol, and propanol. The lactic acid fermentation enhanced the sensory quality, which is related to the amounts of lactic acid, acetic acid and aromatic compounds (Damiani *et al*., 1996). There are two categories of flavour compounds produced during sourdough fermentation. The first category includes non-volatile compounds including organic acids produced by homofermentative and heterofermentative bacteria, which decreases pH, acidifies, and contributes to the aroma of bread dough (Gobbetti *et al*., 1995b). The second category of volatile compounds of sourdough bread includes alcohols, aldehydes, ketones, esters and sulphur. These compounds are produced by biological and biochemical actions during the fermentation process and contribute to the flavour of the final product (Spicher, 1983). Major volatile components in fermented wheat flour include

ethyl acetate, acetaldehyde, hexanal, octanal, nonanal and diacetyl (Damiani *et al*., 1996). However, 1-hydroxy-2-propanone, furfuryl alcohol, pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, hexanal, furfural, pentanol, 3-hydroxy-2-butanone and ethyl-3,6-dimethylpyrazine were the major volatiles of rice flour (Buttery *et al*., 1999). Whereas major volatiles identified in corn flour include 3-methylbutanal, 2-methylpyrazine, 2,5-dimethylpyrazine, 1- hydroxy-2-propanone, 4-vinylguaicol and furfuryl alcohol (Buttery and Ling, 1999).

The quantity of volatile flavour compounds can be improved by the addition of glucose and sucrose; less by maltose. Addition of enzymes to sourdough sponges can also enhance bread volatile compounds (Martínez-Anaya, 1996). Low temperature (25°C) and sourdough firmness are appropriate for LAB souring activities but limit yeast metabolism. Raising the temperature to 30°C causes semifluid sourdoughs to give more complete volatile profiles. At 3h leavening time, the sourdough is mainly characterised by iso-alcohols. An increase of leavening time up to 9h gives a total amount of volatiles about three times higher than that at 5h and strengthens the LAB contribution (Gobbetti *et al*., 1995b). The additions of fructose and citrate to the dough have been reported to enhance the acetic acid and volatile synthesis by LAB, respectively. After baking, the ethanol disappears, 2-methyl-lpropanal is synthetized, lactic and acetic acids remain constant, the total amount of volatiles is reduced to a level <12.5% of the initial, and an increase in the relative percentage of isoalcohols and aldehydes are detected (Gobbetti *et al*., 1995b).

1.10.6 Beneficial effects of sourdough fermentation on bread quality

Sourdough fermented bread can have health benefits to the consumer. It is considered to play a key role in the improvement of overall quality, flavour, texture, nutritional and shelf life properties of bakery products (Katina *et al*., 2005; Carnevali *at el*., 2007; Ryan *et al*., 2008; Gobbetti *et al*., 2014). Figure 1.5 shows a process flow diagram (PFD) for bread making with fermented sourdough in which the sourdough has been kept metabolically active and probably microbially stable for decades by the addition of flour and water at regular intervals. The sourdough is an intermediate, not an end product (Hansen and Schieberle, 2005; Hui, 2006). The microbial activities in the sourdough (mother sponge) have to be judged on the basis of their impact on the quality of baked goods that are produced with its aid. These are characterised by their flavour, nutritional value and texture, i.e. the size and distribution of pores and the elasticity of the bread crumb (Hammes and Gänzle, 1997).

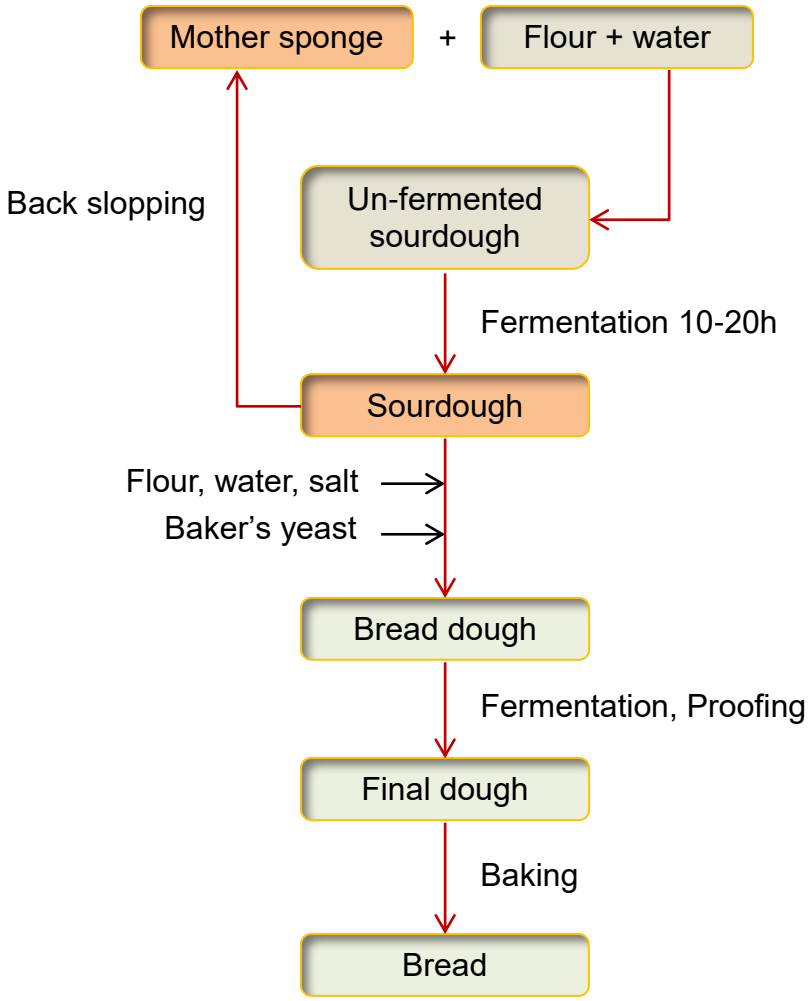


Figure 1.5: Process flow diagram (PFD) of sourdough bread production. Adapted from Hansen and Schieberle (2005) and Hui (2006)

The sourdough fermentation inhibits the growth of the pathogens by synthesizing antimicrobial compounds, like lactic acid, acetic acid, benzoic acid and hydrogen peroxide (Park *et al*., 2006). Health benefits of sourdough fermentation by LAB are based mainly on the production of lactic acid, which subsequently decrease the pH below the point at which undesirable microorganisms can grow (Diowksz and Ambroziak, 2006).

In order to reduce the use of preservatives and treatments that may affect healthy attributes of food, attempts have been made to improve bread quality and shelf life through formulation with compounds naturally occurring in foods (Lavermicocca *et al*., 2000). Gobbetti *et al*. (2005) showed that the presence of sourdough in bread can enhance the digestion process through substantial degradation of cereal components that occurs during fermentation as compared to breads made with baker's yeast leavening.

Liukkonen *et al*. (2003) and Kariluoto *et al*. (2004) reported that sourdough fermentation has caused an increase in folate content; on the other hand, sourdough fermentation has been shown to decrease tocopherol and tocotrienol content (Wennermark and Jägerstad, 1992; Liukkonen *et al*., 2003).

The sourdough has also great potential to modify the macromolecules in the dough, the most well-known examples being the ability of sourdoughs to reduce the digestibility of starch (Liljeberg *et al*., 1995). The presence of lactic acid in bread, either added or formed during sourdough fermentation, has also been reported to reduce acute glyceamic and insulinaemic responses (Liljeberg *et al*., 1995; De Angelis *et al*., 2007).

Sensory evaluation of food and food products is one of the most important aspects of quality control and it is a powerful tool with a wide range of applications in the bakery industry (Elia, 2011). The sourdough fermentation effects the sourdough bread by improving nutritional value and sensory quality (Mueen-Ud-Din, 2009). The optimal use of sourdough can improve the taste and flavour of the bread (Rehman *et al*., 2006).

The sourdough fermentation affects the dough rheology at two levels, in sourdough itself, and in bread dough containing sourdough. In dough,

fermentation decreases elasticity and viscosity, whereas the addition of sourdough to final bread dough results in less elastic and softer dough's. The level of rheological changes taking place in these doughs and its influences on bread quality can be controlled by adjusting fermentation time and the ash content of flour during the pre-fermentation process (Clarke *et al*., 2004).

Di Cagno *et al*. (2002) measured the rheology of fermented dough's by using empirical techniques and found a decrease in resistance to extension and an increase in both extensibility and degree of softening. During the sourdough fermentation different organic acids are produced. These organic acids improve the flavour of bread, help the swelling of gluten and increase gas retention, which results in products with good texture and massive volume and also function as natural dough conditioner (Park *et al*., 2006). LAB are responsible for the aroma and flavour of fermented products. The sourdough wheat bread is more aromatic than simple wheat bread because of its long fermentation time (Brummer and Lorenz, 1991). Sourdough in the baking process has been shown to increase the rate of acidification, improve bread volume and retard bread staling in white wheat bread (Corsetti *et al*., 2000; Di Cagno *et al*., 2003).

There are several functions of sourdoughs which lead to improve bread making as reported by some researchers (Salovaara, 2004; Katina *et al*., 2005; Lahtinen *et al*., 2012; Galle, 2013).

- Leavening action by yeast growing in association with heterofermentative LAB, which make the dough easier to bake, increase bread volume, improve bread-crumb quality, delay staling of bread and increase palatability
- Modification of flour components, such as swelling and partial hydrolysis of protein and polysaccharides, has some advantages, such as improvement

of baking properties of rye dough, improvement of crumb properties of wheat and rye bread, and control of excessive enzymatic activity of rye flour, especially α-amylase and starch degradation in wheat breads by using amylolytic strains

- Occurring organic acids and low pH, and possibly other mechanisms contribute fermentation processes which can control and inhibit contamination or spoiling flora. As result, elongation of mould-free time of bread and prevention of growth of *Bacillus subtilis* and the rope-causing organism can be obtained
- Bread flavour and aroma are built up as a result of accumulation of organic acids such as lactic and acetic acids which are produced by LAB and their reaction with other dough components such as alcohol
- Accumulation of flavour precursor compounds such as amino acids and reducing carbohydrates
- Sourdough fermentation has a well-known role in improving the nutritional properties of wheat, rye and oat baked goods via increasing the levels of bioactive compounds such as phytochemicals in cereals. These health-beneficial phytochemicals include lignan, phenolic acids, phytosterols, tocos, folates and other vitamins that are found concentrated in the germ and the outer layer of kernel
- Phytic acid is degraded by phytase in flour and from LAB. This increase the bioavailability of iron and other minerals
- Identification of the product by a natural image; greater versatility, local and regional products

- Lower glycemic index values of wheat bread can be obtained through modification of starch structure by using amylolytic strains of LAB which can break down starch into glucose. Inversely, starches with lower amylose content can have higher glycemic indexes

1.11 The rationale

The interest in consuming natural foods, with the least possible amount of chemical additives to be found, is increasing. Consumers have become more demanding about the quality of food. Research has shown that LAB for fermentation of bread products could increase the shelf life and microbiological safety of bread products. Also, there are lots of potential advantages in improving the quality, sensory characteristics, texture and acidity of the fermented bread products by LAB. Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of “natural” and “traditional” foods, processed without any addition of chemical preservatives, are becoming more attractive.

There are several advantages of acidic food fermentation; first of all, it increases food’s resistance to microbial spoilage and the development of food toxins, decreases pathogenic microbial activity in the food, preserves foods between the time of preparing and consumption, modifies the flavour of the products, and also improves nutritional value.

Bread is one of the most important products as a balanced diet in many parts of the world, especially in the developing countries. Today, many types of bakery products can be found on market shelves. Most bread products have a short

shelf life when stored at room temperature. Crumpets are a type of bread products which can be stored at ambient temperature, having a short shelf life which is usually preserved by chemical additions. Buttermilk is often used in baking because of its special properties. Fermented buttermilk is a great source of vitamins like vitamin B complex, high levels of proteins, natural enzymes, calcium, and potassium and probiotic bacteria and their metabolites compounds (Chandan *et al*., 2008). The first experiment (Chapter 3) is conducted with four types of buttermilk fermented with *Lactococcus lactis* subsp. *lactis* and using commercial nisin additive. Chapter three investigates the antimicrobial activities of them *in vitro* against some pathogenic bacteria strains. Studies on the addition of fermented Buttermilk (FBM) and nisin-producing *Lc. lactis* subsp. *lactis* on bread crumpets would present an opportunity to preserve it without using chemical preservatives. Moreover, they could give very useful information about the influence of them on the quality, delay staling and the shelf life of the final bread product (Chapter 4).

Cereal-based foods represent a very important source of biological as well as cultural diversity. Sourdough has proven to be useful in improving dough properties; bread texture and flavour, delaying the staling process and preventing bread from spoilage. There are many studies conducted on the sourdough diversity especially in European countries. However, there are not as many available studies on the sourdough diversity in the UK as there are in the rest of Europe. Studying sourdough aims to investigate the biodiversity and find starter cultures from the spontaneously fermented sourdough collection. They would help for sourdough fermentation and can also be used for bread-making (Chapter 5 and 6). The use of fermented sourdough by LAB may have

an influence on the bread quality and shelf life and functionality of bread products by inhibiting or killing undesirable pathogenic microorganisms via a number of metabolites compounds, which may be produced by LAB such as the production of lactic acid and antimicrobial peptides (bacteriocins) etc. In addition, it may potentially affect the nutritional value and sensory properties of bread products.

1.12 The aim and objectives of this study

The overall aim of this study is to investigate the effect of LAB and their bacteriocins on the shelf life of cereal products, with the purpose of reducing the chance for pathogenic microorganisms to grow on the product during the storage period. The schematic outline of the thesis is summarised in Figure 1.6.

The specific objectives of this study are addressed as the followings:

- To use LAB and their bacteriocins as an alternative to chemical preservatives which are assumed to have side effects on health
- To evaluate the influence of LAB and their bacteriocins as bio-preservatives for bread products (crumpets and sourdough bread) to prolong shelf life and increase safety of bread products through the fermentation process
- To evaluate the influence of LAB and their metabolites on the acidification values of bread products (crumpets and sourdough bread) through the fermentation process
- To investigate the potential modification changes to bread texture and quality after treatments
- To investigate the effects of LAB and their metabolites on the sensory attributes of bread products
- To investigate the effects of LAB and their metabolites on the colour changes of bread products (crumpets and sourdough bread)
- To assess the ability of LAB and traditional fermentation to kill or restrict the growth and proliferation of pathogenic and spoilage microorganisms, to improve safety of products

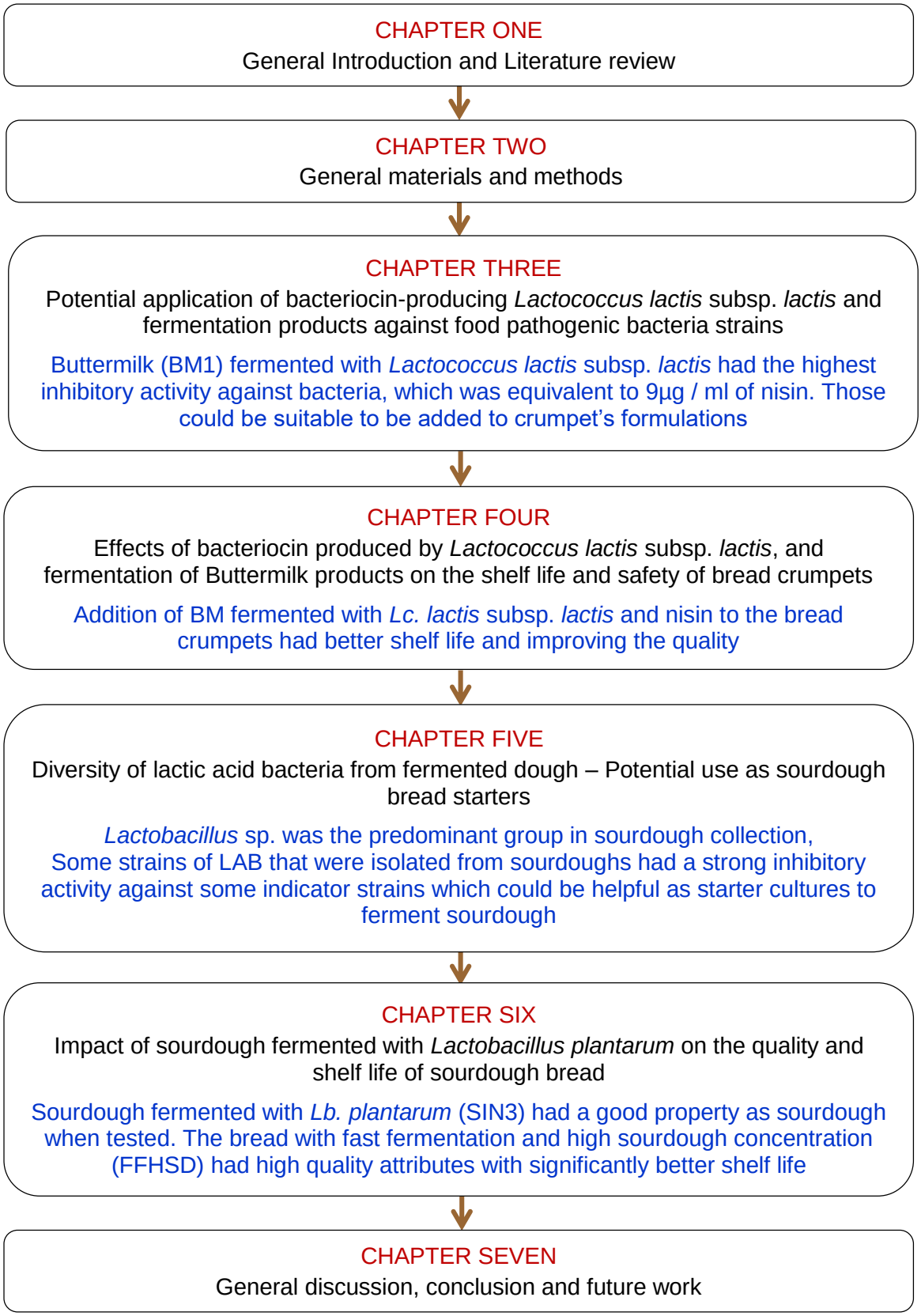


Figure 1.6: A schematic outline of the thesis with the main outcomes

CHAPTER TWO

General materials and methods

2.1 Methods

This chapter includes the general materials and methods which were applied to four experiments. All experiments were carried out in the microbiology and nutrition laboratories at the University of Plymouth. The general procedures and analytical techniques, which were used in the present study, are presented in this chapter. Other techniques specific to particular trials are described in the relevant experimental chapters. In the first experiment, four types of fermented buttermilk (BM) products with *Lactococcus lactis* subsp. *lactis* and commercial nisin (3, 6, 9, 12µg/ml) as a reference, were examined *in-vitro* against some pathogenic bacteria to select the best fermented BM and commercial nisin were used as bio-preservatives for bread crumpets. The second experiment was conducted to use fermented BM with *Lc. lactis* subsp. *lactis* and nisin additive (in the first experiment) for adding bread crumpets, to investigate the safety and quality changes and also extending the shelf life of bread crumpets.

The last two experiments were conducted to characterise fermented sourdough to be used for making bread. Purpose to improve the quality changes, increasing the safety and shelf life of bread products and also for evaluation of sensory characteristics. Sourdough and bread samples (n=18) were collected in the third experiment to assess the diversity of LAB isolate strains which would be useful for making bread with acceptable dough properties and extending the shelf life of bread. The fourth experiment was investigated on the properties of

sourdoughs preparation to select the best sourdough for making sourdough bread with acceptable properties and longer shelf life of bread.

2.2 Bacterial strains

The following strains were obtained from the School of Biological Sciences/ culture collection (microbiology lab) at the University of Plymouth : *Lactococcus lactis* subsp. *lactis* ATCC 8656, and the following indicator strains used for the inhibitory activity test, *Bacillus cereus* NCIMB 11925*, Bacillus subtilis*, *Pseudomonas aeruginosa* ATCC 10817*, Escherichia coli* ATCC 10418 and *Staphylococcus aureus* ATCC 6821.

2.3 Culture media

Unless otherwise indicated, media were prepared according to the manufacturer's instructions and were sterilised by autoclaving at 121°C for 15min.

2.3.1 Nutrient broth and agar

Nutrient broth (CM0001, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by dissolving 13g of base in one litre of distilled water then distributing in 10ml aliquots into universal tubes, using an Eppendorf dispenser (Eppendorf AG, 22331, Hamdarg, Germany) and autoclaved at 121°C for 15min. 15g/litre agar (LP0011, Oxoid Ltd., Basingstoke, Hampshire, England) was added to the media before autoclaving to prepare of nutrient agar.

2.3.2 Brain heart infusion agar (BHI)

BHI agar (CM1135, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by dissolving 37g of base in one litre of distilled water microwaving for 10min, then distributed in 20ml aliquots in universal tubes. All tubes were sterilised by autoclaving for 15min at 121ºC and used to prepare agar plates for the agar well diffusion method.

2.3.3 *Bacillus cereus* selective agar base medium

Bacillus cereus selective agar base medium (CM0617, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by suspending 20.5g in 475ml of distilled water. Then microwaving for 10min and sterilised by autoclaving at 121°C for 15min. The medium was cooled to 50°C, one vial of polymyxin B supplement (SR0099) and 25ml of sterile egg yolk emulsion (SR0047) was added to the media and then it was mixed well and poured into sterile Petri dishes.

2.3.4 M17

M17 (CM 0817, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by dissolving 37.25g of base in 950ml of distilled water, then the media was sterilised by autoclaving for 15min at 121ºC. After autoclaving and cooling the media to 50ºC, 50ml of lactose solution (10% w/v) sterilised by membrane filtration through 0.2µm were added to M17 broth.

2.3.5 Preparation of De Man, Rogosa Sharpe agar (MRS)

MRS (CM 0817, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by dissolving 60.5g of base in one litre of distilled water and adding one ml tween 80, and then the media was sterilised by autoclaving for 15min at 121ºC.

2.3.6 Potato Dextrose agar (PDA)

PDA (CM0139, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared according to the manufacturers' instructions by dissolving 25g of base in one litre of distilled water then the media was autoclaving for 15min at 121ºC and was used for determination of moulds and yeasts.

2.3.7 Yeast extract glucose chloramphenicol agar

The medium used for determination of yeast from sourdough fermentation was prepared by mixing yeast extract (5g), glucose (20g), Chloramphenicol (0.1g) and agar 15g with one litre of distilled water. The pH of Media was adjusted to 6.6±0.2 then it was sterilised by autoclaving at 121°C for 15min.

2.3.8 Plate count agar (PCA)

PCA (CM 0325, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared by dissolving 17.5g of base in one litre of distilled water, then the media was sterilised by autoclaving at 121ºC for 15min. This media was used for spore forming bacteria.

2.4 Preparation of standard (McFarland's) opacity tube

A specific amount (0.05, 0.1, 0.2, 0.3 and 0.4)ml of 1% barium chloride was added to 1% sulphuric acid (9.95, 9.9, 9.8, 9.7 and 9.6)ml respectively in order to prepare (0.5, 1, 2, 3 and 4) McFarland opacity standard tubes (Harrigan, 1998).

2.5 Preparation of saline solution

Saline Tablets BR0053 (Oxoid Limited, Basingstoke, Hampshire, England) were used by dissolving one tablet in 500ml of distilled water to obtain 0.85% saline solution and used to dilute the bacterial cultures.

2.6 Preparation of inoculum bacteria

A single colony of inoculum was taken from colonies grown previously as a pure culture on a plate and inoculated into 10ml nutrient broth. The broth suspension was incubated for 18h at 37°C (LEEC incubator, LEEC Limited, Nottingham, UK), except *Pseudomonas aeruginosa* which was incubated at 30°C (Swallow Incubators, LTE Scientific Ltd, Oldham, UK. Bacterial cultures were diluted by using saline solution and were standardised to 10^7-10^8 CFU/ml using the McFarland standards by visually comparing the opacity of the bacterial suspension to the 0.5 McFarland standard.

2.7 Measurement parameters of bread samples

Crumpets and sourdough bread were prepared for separate experiments. The procedures for bread making are each presented in this chapter. Nevertheless, some parameters were obtained using the same procedures in both cases such as pH and acidity values, colour measurement, water activity and microbial shelf life of bread products.

2.7.1 Measuring PH value

The pH value of samples was determined by mixing 10g of the sample with 100 ml of distilled water and subsequently homogenised for 3min in a stomacher (Bag mixer 100 MiniMix, Arpents, France). pH meter (pH 213, HANNA Instrument, Indonesia) was used for measuring a pH value which was previously calibrated (pH 4.0 and 7.0). A pH electrode was then flooded directly into the sample. The pH values were recorded in triplicate samples (El-Khoury, 1999).

2.7.2 Measuring titratable acidity value

The suspension of the sample was prepared for pH value as mentioned previously was titrated against 0.1N NaOH with phenolphthalein indicator. Total titratable acidity (TTA) was expressed as the amount of NaOH was used (in ml). Triplicate of TTA was measured from each sample. Titratable acidity (TA) was expressed as lactic acid percent (mg/100mg) according to standard procedure as described by AACC method 02.31 (2000).

$$\text{TA (mg/100mg)} = \frac{\text{NaOH (in ml)} \times 0.1\text{N} \times \text{Equivalent weight of lactic acid (90.08)}}{\text{Weight of sample (g)} \times 10}$$

2.7.3 Water activity measurement for bread samples

Water activity (Aw) for samples was determined in triplicate at room temperature (22°C) using a Aw meter (Novasina Thermoconstanter, TH-2/RTD-33, Zürich, Switzerland). Samples were transferred to a measuring cell until about half full, and then the measuring cell was left in the instrument until constant readings indicated that equilibrium was reached.

2.7.4 Measuring the colour of bread samples

The colour of bread crumb and crust was measured using a Minolta colorimeter (Minolta Ltd.; Model, CM2600d, UK) with a 10° standard observer and D65 (room light) and calibrated with a white plate standard. The colour of sample was denoted by the 3 dimensions *L**, *a**, and *b**. When the *L** scale represents the value of a product lightness from zero (for dark) to 100 (absolute white). Components *a** and *b** represent redness/greenness and yellowness/blueness colour of products respectively. Nine replicates were run per sample and the whiteness was calculated according to the following equation (Hsu *et al*., 2003; Chiavaro *et al*., 2008; Borsuk *et al*., 2012).

$$\text{Whiteness} = 100 - [(100-L^*)^2 + a^{*2} + b^{*2}]^{0.5}$$

2.7.5 Microbial shelf life determination of bread samples

The bread samples were taken for microbiological analysis every two days during the storage period until microbial growth was observed on the surface of bread samples. Ten g of sample was homogenised with 90 ml of PBS buffer solution (0.1 M, pH 7.0) for 3min in a stomacher (Bag mixer 100 MiniMix, Interscience, Arpents, France). Aliquots were serial diluted in maximum recovery diluent and plated out following National Standard Methods (HPA, 2004, 2009). Aerobic plate count (APC) were determined on nutrient agar. The inoculated nutrient agar was incubated at 37ºC for 48h. LAB counts were determined on MRS Agar medium, with the inoculated plates incubated under 5% CO_2 incubator at 37°C for 48 hours. Mould and yeast counts were enumerated on potato dextrose agar medium, and then the plates were incubated at 25°C (±2°C) for 5 days.

Bacillus cereus was enumerated on *Bacillus cereus* selective agar base medium with polymyxin B supplement (SR0099) and egg yolk emulsion (SR0047) to detect the growth of *Bacillus cereus* in bread samples, the inoculation media were incubated at 37ºC for 48h (Ntuli *et al*., 2013).

For the spore forming bacteria, plate count agar medium was used to determine the number of spore forming bacteria. First, the dilution of the bread samples was heated to 80ºC for 10min. Then the inoculated plates were incubated at 37°C (±2°C) for 48h (Stear, 2012).

2.8 Sensory evaluation

The sensory evaluation protocol was approved by the Human Ethics review committee of the Faculty of Science and Engineering, Plymouth University. Information and a consent form stated that each participant could withdraw from the panel at any time during the experiment.

Bread samples were subjected to sensory evaluation by 33 non-expert panellists (Plymouth University students and staff members of) the day after baking. Each bread sample was sliced into 3cm thickness and evaluated on the basis of the following characteristics; overall appearance, Aroma, texture, flavour, acidity, and overall acceptability. The scoring scales for each attribute were as follows; 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like or dislike, 6= like slightly, 7= like moderately, 8= like very much and 9= like extremely.

2.9 Statistical Analysis

All data were subjected to one way analysis of variance (ANOVA) using Minitab statistics software version 16.0 (Minitab, Coventry, UK) to compare different treatment groups followed by Tukey's Multiple Comparison test. Data are presented as mean ± standard deviation (SD) and $P<0.05$ was considered significant. Sensory evaluation data is presented as average ranks, and whenever conditions for ANOVA were not met (e.g. not normally distributed), a Kruskal – Wallis test (Non parametric) with Dunn's test was used to determine significant differences between the different parameters as suggested by O'Mahony (1986).

CHAPTER THREE

Potential application of bacteriocin-producing *Lactococcus lactis* subsp. *lactis* and fermentation products against food pathogenic bacteria strains

3.1 Introduction

LAB and their metabolites commonly have a significant role in the preparation and bio-preservation of food products which are due to their contributions to flavour, aroma, prolonging shelf life and increasing safety of food products by inhibiting growth of food spoilage and food pathogen microorganisms (Devlieghere *et al*., 2004; De Vuyst and Leroy, 2007). In previous studies, LAB have been used as biopreservatives to extend shelf life of food products e.g. bread products (Ogunbanwo *et al*. 2008; Saranraj and Sivasakthivelan, 2016), beef steaks (Djenane *et al*., 2005) and processed sliced apples and lettuce with *Lactobacillus plantarum* (Siroli *et al*., 2015). An example of the shelf life of bread reported by Axel *et al*. (2015) who found that the shelf life of the Quinoa breads (gluten-free sourdough bread) containing *Lb. amylovorus* fermented sourdough increased for 4 days compared to the non-acidified control. LAB are an important microorganism in traditional industrial field that have the fermentative ability in food products and also recognized as healthy and beneficial organisms (Gilliand, 1990; Caplice and Fitgerald, 1999). LAB have been used as a natural food preservative to increase safety and quality of food products and also have effect on food stability. They have been used to preserve bread products for longer shelf life, delay staling and may also minimise the changes in physico-chemical properties of products during storage (Brul and Coote, 1999; Onilude *et al*., 2005; Plessas *et al*., 2008).

Lactococcus (*Lc*.) strains have been used for centuries as starters for the manufacture of cheeses and other fermented dairy products. *Lc. lactis* strains (particularly *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris*) have been used as commercial starter cultures in industrial fermentation for dairy products e.g. hard cheeses (Hutkins, 2006). On the other hand, food producers face a major challenge in an environment in which consumers demand safe foods with a long shelf life, but also express a preference for minimally processed products that do not contain chemical preservatives. Bacteriocins present an attractive option that could become at least part of the solution. They are produced by food-grade organisms, are usually heat stable, and have the ability to inhibit many primary pathogenic and spoilage microorganisms (Chen and Hoover, 2003; Ouwehand and Vesterlund, 2004; Von Mollendorff *et al*., 2006).

Lc. lactis is a Gram-positive bacterium used extensively in the production of buttermilk and cheese. It is an obligately homofermentative, facultative anaerobe, with an optimum growth temperature near 30°C. *Lc. lactis* is among the most important of all LAB (and perhaps one of the most important organisms involved in food fermentations, period). *Lc. lactis* is known as generally recognized as safe (GRAS) status (Wessels *et al*., 2004; Hutkins, 2006). *Lc. lactis* subsp*. lactis* BZ isolated from boza and its bacteriocin has a wide inhibitory activity against several Gram-positive and Gram-negative foodborne pathogens and food spoilage bacteria e.g. *B. cereus*, *B. subtilis*, *E. coli*, *Listeria monocytogenes* and *Salmonella* sp. and it can be potential for use as a bio-preservative in food products (Şahingil *et al*., 2011).

Nisin is a natural antimicrobial polypeptide produced by *Lc. lactis* subsp. *lactis*. It belongs to the group of inhibitors called bacteriocins (Thomas and Delves-

Broughton, 2005) and it effectively inhibits Gram-positive bacteria and also the outgrowth of spores of *bacilli* and *clostridia* (Gandhi and Chikindas, 2007). The application of nisin as a food preservative has been studied extensively (Marth, 1966; Lipinska, 1977; Hurst, 1981; Hurst and Hoover, 1993).

Buttermilk is a diary product and it is widely used in the food industry by many people around the world for centuries, because of emulsifying capacity and its positive effect on flavour (Sodini *et al*., 2006). Buttermilk is a by-product, a liquid left over after extracting butter from churned yoghurt and cream (Jinjarak *et al*., 2006; Costa, 2010). Buttermilk contains all the water soluble component of cream such as milk protein, lactose and minerals. It also includes materials like phosphotidyl choline (lecithin) derived from milk fat globule membrane which is disrupted during the churning and mostly migrates to the buttermilk fractions. Many kinds of buttermilk preparation methods are available in the world. Dry buttermilk is the product resulting from the removal of water from liquid buttermilk (Chandan *et al*., 2008). Some of the lactose of buttermilk is converted into lactic acid by the LAB, which gives the milk a slightly sour taste and makes it easier to digest by lactose intolerant consumers (Chandan *et al*., 2008). The use high concentration of salt, sugars and chemical addition for preserving food products which might be affecting the quality and nutritional value of the food products that causes health problems as well (Uhlman *et al*., 1992; Kelly *et al*., 1996).

The aims of this study were:

- To investigate the antimicrobial activities of four types of buttermilk fermented with *Lc. lactis* subsp. *lactis* and commercial nisin as a reference *in vitro* against some pathogenic bacteria strains
- To select the best buttermilk fermented with *Lc*. *lactis* subsp. *lactis* and commercial nisin as a reference for further investigation in this study

3.2 Materials and methods

3.2.1 Bacterial strains, culture media and preparation of inoculum bacteria

The bacterial strains were obtained from the School of Biological Sciences / University of Plymouth culture collection as detailed in Section 2.2. Nutrient broth, BHI and M17 were prepared as described in Section 2.3.1, 2.3.2 and 2.3.4 respectively. Inoculum bacteria were prepared and standardised as described in Section 2.6.

3.2.2 Nisin products

Commercial nisin in the form of Nisaplin (PD214210-7.2EN) was obtained from Danisco Company (Danisco A/S DK, Denmark) which is produced by *Lc. lactis* subsp. *lactis* and it was used as a preservative for food product to increase quality and shelf life of food products.

3.2.3 Buttermilk products

Four different buttermilk (BM) were studied, including three commercial buttermilk powders which are Frontier buttermilk powder (BM1) (Frontier, Norway, IA, USA), Now real food buttermilk powder (BM2) (Now foods Bloomingdale, IL, USA) and Bob's red mill buttermilk powder (BM3) (Bob's red mill natural foods SE Pheasant Court Milwaukie, Oregon, USA), Components are shown in the Table 3.1. The other buttermilk (BM4) was churned from cream then it was frozen.

Table 3.1: Commercial buttermilk powder's components

Components per 120g	Frontier buttermilk powder (g)	Now Real Food buttermilk powder (g)	Bob's Red Mill buttermilk powder (g)
Sugar	56	60	56
Fat	6	8	8
Protein	40	40	40
Sodium (mg)	0.60	0.60	0.68
Total	102.6	108.6	104.68

3.2.4 Buttermilk preparation

Three commercial buttermilk powders were prepared in three different ways as shown in Figure 3.1. The first one was prepared according to manufacture and referred as control that was prepared by adding 120g in 480ml water (BM1), 120g in 1200ml water (BM2) and 120g in 1680ml water (BM3). Other preparations were standardised on sugar content (46.66%) and solid content

(85.5%) of BM1, and the frozen buttermilk was used directly after churned from cream.

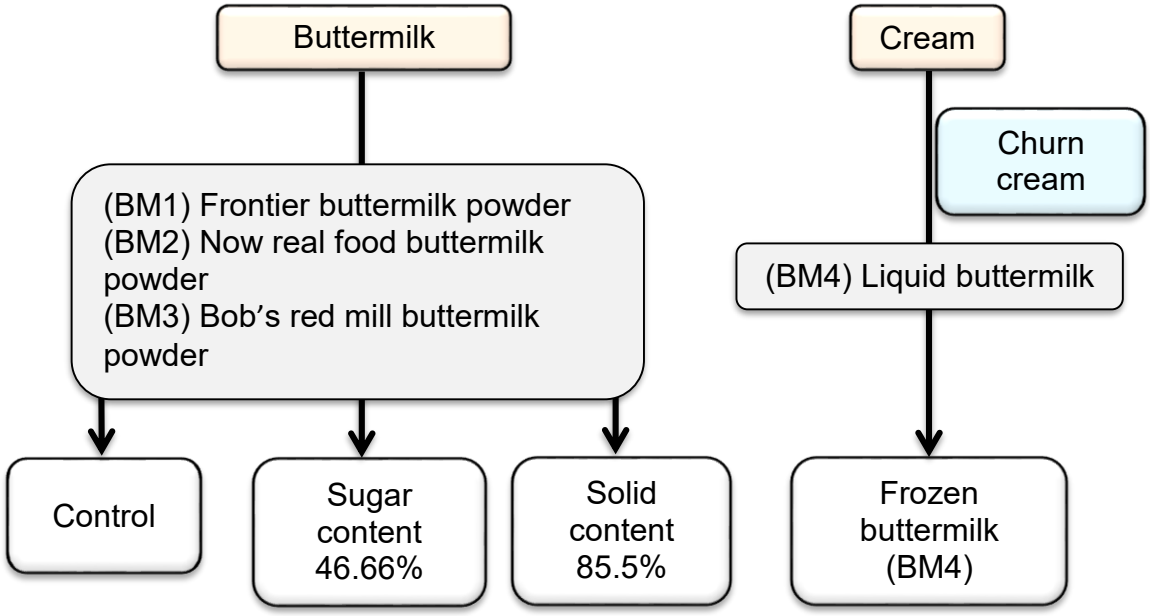


Figure 3.1: buttermilk preparation design

3.2.5 Preparation of *Lc. lactis* subsp. *lactis*

Lc. lactis subsp. *lactis* was cultured at 30°C overnight in M17 broth supplemented with 0.5% lactose (Terzaghi and Sandine, 1975; Cheigh *et al*., 2002). Resulting *Lc. lactis* subsp. *lactis* was inoculated (1% by volume) into 100ml M17 broth, and then incubated for 24h at 30°C. The cells were harvested by centrifugation at 10000rpm for 10min (Rotina 46 centrifuge, Hettich Zentrifugen, Germany) and washed twice with sterilised saline solution (Kivanç, 1990) after that the cell was suspended in 10ml of each prepared buttermilk separately. The cells were incubated at 30°C for 24h, the level of cell was 10^9 CFU/ml and then it was used for subsequent studies.

3.2.6 pH and titratable acidity (TA) of buttermilk products

The pH and TA values of buttermilk products were measured directly after 0, 8, 16 and 24h as detailed in Section 2.7.1 and 2.72 respectively.

3.2.7 Preparation of Cell free supernatant

Cell free supernatant (CFS) was prepared from each buttermilk fermented with *Lc. lactis* subsp. *lactis*. CFS was obtained by centrifuging the culture at 10000rpm for 10min. CFS was sterilised by membrane filtration through 0.22µm pore size filter (Milipore Ireland Ltd, Cork, Ireland).

3.2.8 Agar well diffusion bio-assay for activity of buttermilk products fermented with *Lc. lactis* subsp. *lactis*

The agar well diffusion method was used to determine the antimicrobial activity of *Lc. lactis* subsp. *lactis* against bacterial strains such as *B. cereus*, *P. aeruginosa*, *E. coli* and *S. aureus* (Kuri *et al*., 1998; Fernández-López *et al*., 2005). Stock cultures of all tested bacteria were grown in nutrient broth for 18h. Final cell concentrations were standardised to 10^7-10^8 CFU/ml using the McFarland standards as mentioned in Section 2.6. Then, 200µl of this inoculum was added to each universal tube containing 20ml molten brain heart infusion (BHI) agar, mixed well and poured into a disposable Petri dish. A sterile cork borer was used to make wells (5mm diameter) after the agar was solidified. Forty µl of fermented buttermilk supernatants was added into each well and was left to diffuse for one h at room temperature, then incubated at 37°C for 24h.

After incubation, the diameter (mm) of the inhibition zone around the wells was measured in three directions using Vernier callipers and the averages were calculated (Kuri *et al*., 1998). The assay was carried out in triplicate.

3.2.9 Determination of the antimicrobial activities of buttermilk supernatant and commercial nisin against food pathogenic strains

In this method 96-well micro plate (Thermo Scientific, Nunclon Delta Surface, Denmark) was used to determine cells densities of pathogenic bacterial strains by adding supernatant of fermented buttermilks at manufacture preparation and different concentration of commercial nisin. This method was used to compare the antimicrobial activity of them against the same food pathogenic bacterial strains. 1% of buttermilk supernatant was suspended in BHI broth separately and also nisin was suspended in BHI broth at different concentration from 3, 6, 9 and 12µg/ml with control. Mainly 90µl of prepared antimicrobials from each BHI broth was pipetted into the wells of sterile micro plate and 10µl of 10^7-10^8 CFU/ml culture of each overnight bacterial test was inoculated in wells, Then micro plate kit was incubated in a micro plate reader (Tecan Infinite M200 Pro Microplate Reader, Tecan Austria GmbH) at 37°C for 24h, the growth of each strain was determined by measuring the optical density (OD) at 595nm. Three replications were made for the experiment.

3.2.10 Detection of nisin activity and fermented buttermilk against spores of *Bacillus cereus*

The method is described by Jenson *et al*. (1994) in which spores of *B. cereus* were tested for their sensitivity to nisin. *B. cereus* was grown in nutrient broth at 37°C for 72h, and then the growth culture was heated to 80°C for 10min. The spore suspension was diluted in sterile ringer solution and 0.1ml of each dilution was transferred into nutrient broth, which was suspended with 1% of each fermented buttermilk supernatant and different concentration of nisin from 3, 6, 9 and 12µg/ml with control. The broth cultures were then incubated at 37°C for 7 days. The control tube containing no fermented buttermilk and no nisin was used to estimate the number of spores added to each tube. The highest dilution for which growth was noted, was deemed to have 10spore/ml. The measure of the presence or absence of growth was noted. Three replications were made for the experiment.

3.2.11 Determination of the antimicrobial activities of bio-preservatives and chemical preservatives against bacterial tested

A 96-well micro plate (Thermo Scientific, Nunclon Delta Surface, Denmark) was used to determine cells densities of bacterial cells tested by adding various antimicrobial activities which are included commercial nisin, potassium sorbate, calcium propionate and vinegar to BHI broth at different concentration. Commercial preservative nisin was added in the form of nisaplin into BHI broth by different concentration including 0, 3, 6, 9 and 12µg/ml. Potassium sorbate, calcium propionate and vinegar were added into BHI broth 0, 100ppm, 300ppm,

600ppm and 900ppm. The acidity of malt vinegar was 5% (v/v) (Sarson's malt vinegar, UK).

For each prepared antimicrobial 90µl were pipetted into the wells of sterile micro plate and 10µl of 10^7-10^8 CFU/ml culture of each overnight bacteria were inoculated in wells. Then, micro plate was incubated in a micro plate reader at 37°C for 24h, the cells densities of each strain were determined by measuring the optical density (OD) at 620 nm. Three replications were made for the experiment.

3.2.12 Statistical Analysis

All data were analysed statistically as detailed in Section 2.9.

3.3 Results

3.3.1 pH and TA values of fermented buttermilk

pH and TA values of different types and prepared in three different methods of fermented buttermilk products are shown in Tables 3.2 and 3.3 respectively.

The pH values of all types and preparation methods of buttermilk ranged between 6.67 and 6.69 before incubation period of buttermilk products. During incubation periods, the pH values of fermented buttermilks was decreased significantly (P<0.05) according to the type of buttermilk products and preparation methods. After 24h incubation period, there were significant differences in pH levels between fermented buttermilks of up to 4.92 to 5.39 according to the type of buttermilk products and preparation methods. During time point of fermented buttermilks, the pH of fermented BM1 at each preparation method was lower than the other fermented buttermilk products. The pH value of churned BM was higher which maight be due to of the frozen BM was used (Table 3.2).

Before incubation period, the TA values of buttermilks ranged between 0.66 and 0.73. During incubation, the TA value of fermented buttermilks was significantly increased (P<0.05) according to the buttermilk (BM1-BM4) and preparation methods. Additionally, there were significant differences in TA between fermented buttermilks. The TA value of BM1 was higher than the other fermented buttermilk at each preparation method in each time point of fermented buttermilks (Table 3.3).

Table 3.2: pH development* of buttermilk (BM1-BM4)[a] prepared by three different methods[b] and fermented with *Lc. lactis* subsp. *lactis* over 24h at 30°C

Fermented buttermilk		Time (h)			
preparation	Brand	0	8	16	24
Control	BM1	6.68±0.01 ^{ab}	5.98±0.01 ^{g}	5.36±0.02 ^{f}	4.92±0.01 ^{c}
	BM2	6.67±0.01 ^{b}	6.02±0.02 ^{f}	5.54±0.02 ^{e}	5.08±0.02 ^{b}
	BM3	6.68±0.01 ^{ab}	6.14±0.01 ^{d}	5.83±0.01 ^{b}	5.37±0.01 ^{a}
Fixed sugar content 46.66%	BM1	6.68±0.01 ^{ab}	5.98±0.01 ^{g}	5.37±0.01 ^{f}	4.93±0.02 ^{c}
	BM2	6.69±0.01 ^{a}	6.18±0.01 ^{c}	5.63±0.01 ^{d}	5.11±0.01 ^{b}
	BM3	6.68±0.01 ^{ab}	6.31±0.01 ^{a}	5.81±0.02 ^{bc}	5.38±0.03 ^{a}
Fixed solid content 85.5%	BM1	6.68±0.01 ^{ab}	5.98±0.01 ^{g}	5.37±0.01 ^{f}	4.93±0.01 ^{c}
	BM2	6.67±0.01 ^{b}	6.07±0.01 ^{e}	5.61±0.01 ^{d}	5.10±0.02 ^{b}
	BM3	6.67±0.01 ^{b}	6.24±0.01 ^{b}	5.78±0.02 ^{c}	5.35±0.02 ^{a}
Churned buttermilk	BM4	6.68±0.00 ^{ab}	6.27±0.01 ^{b}	6.86±0.02 ^{a}	5.39±0.04 ^{a}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-g}$ Means in the same column with different superscripts are significantly different (P<0.05).

[a] BM1 is Frontier buttermilk powder; BM2 is Now Real Food buttermilk powder; BM3 is Bob's Red Mill buttermilk powder and BM4 is frozen buttermilk.

[b] Control: commercial products were prepared according to manufacturer's instructions, sugar content: buttermilk powders were standardised on sugar content of BM1, solid content: buttermilk powders were standardised on total solid content of BM1 and churned buttermilk: buttermilk was churned from cream and then frozen it.

Table 3.3: Development of TA values* (mg/100mg) of buttermilk (BM1-BM4) prepared by three different methods and fermented with *Lc. lactis* subsp. *lactis* over 24h at 30°C

Fermented Buttermilk**		Time (h)			
preparation	Brand	0	8	16	24
Control	BM1	0.73±0.01 ^{a}	0.78±0.01 ^{a}	0.85±0.01 ^{a}	0.92±0.01 ^{a}
	BM2	0.72±0.01 ^{ab}	0.75±0.01 ^{b}	0.79±0.01 ^{b}	0.85±0.01 ^{b}
	BM3	0.69±0.02 ^{abc}	0.72±0.01 ^{c}	0.75±0.01 ^{cd}	0.79±0.01 ^{cd}
Fixed sugar content 46.66%	BM1	0.73±0.01 ^{a}	0.78±0.01 ^{a}	0.84±0.01 ^{a}	0.91±0.01 ^{a}
	BM2	0.70±0.01 ^{abc}	0.73±0.01 ^{bc}	0.76±0.01 ^{cd}	0.80±0.02 ^{cd}
	BM3	0.68±0.01 ^{bc}	0.72±0.01 ^{c}	0.74±0.01 ^{e}	0.78±0.005 ^{de}
Fixed solid content 85.5%	BM1	0.72±0.02 ^{ab}	0.77±0.01 ^{a}	0.84±0.01 ^{a}	0.92±0.02 ^{a}
	BM2	0.68±0.01 ^{bc}	0.74±0.01 ^{bc}	0.77±0.01 ^{bc}	0.84±0.01 ^{bc}
	BM3	0.66±0.01 ^{c}	0.68±0.01 ^{d}	0.71±0.01 ^{f}	0.74±0.01 ^{e}
Churned buttermilk	BM4	0.69±0.01 ^{abc}	0.72±0.01 ^{c}	0.75±0.01 ^{de}	0.80±0.01 ^{cd}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a\text{-}f}$ Means in the same column with different superscripts are significantly different (P<0.05).

** Key of the table as Table 3.2

3.3.2 Antibacterial activity of fermented buttermilk

The antibacterial activity of three types with three different methods of preparation of buttermilk and churned buttermilk which are fermented with *Lc. lactis* subsp. *lactis* were tested against some strains of food pathogenic bacteria such as *B. cereus, E. coli*, *P. aeruginosa* and *S. aureus* by agar well diffusion method the result are shown in Table 3.4. The fermented buttermilks gave zones of inhibition against the strains of food pathogenic bacteria strains. All fermented buttermilks were able to inhibit the growth of *B. cereus* and *S. aureus* to varying degrees from 6-16mm except churned BM which had no inhibitory activity against *S. aureus*.

All fermented BM exhibited different inhibitory activity depending on the type and preparation method of the BM as well. Among the four fermented buttermilks, the strongest (14-16mm) diameter zones obtained with the fermented BM1 at all preparation methods against *B. cereus*, and *S. aureus*. Fermented BM1 had an intermediate (10-13mm) diameter zones against *E. coli* and *P. aeruginosa.* Smallest or weak (6-9mm) diameter zones was observed from some other fermented buttermilks (Table 3.4). However, fermented BM3 at all preparation methods and BM4 had no inhibitory activity against *E. coli* and BM4 also with *S. aureus*. Fermented BM2 at sugar content and solid content had no inhibitory activity against *P. aeruginosa*.

Table 3.4: Antibacterial activity* of fermented buttermilk preparations against bacterial food pathogens using an agar well diffusion method

Fermented Buttermilk**		Target strains			
preparation	Brand	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. aureus*
Control	BM1	+++	++	++	+++
	BM2	++	++	+	++
	BM3	++	-	+	+
Fixed sugar content 46.66%	BM1	+++	++	++	+++
	BM2	++	+	-	++
	BM3	++	-	+	+
Fixed solid content 85.5%	BM1	+++	++	++	+++
	BM2	++	+	-	+
	BM3	+	-	+	+
Churned buttermilk	BM4	+	-	++	-

* Diameter of inhibition zone: (–) no inhibition zone, (+) weak (6 – 9mm), (++) intermediate (10 – 13mm) and (+++) strong (14 – 16mm). Mean values from three replicates

** Key of the table as Table 3.2

3.3.3 Effects of fermented buttermilk supernatant and nisin against strains of food pathogenic bacteria strains

The microbial growth (OD_{595}) of the four food pathogenic bacteria in BHI broth media containing four different types of fermented BM supernatant and different concentration of commercial nisin (3, 6, 9, 12µg/ml) as reference and control without additives after 24h of incubation are shown in Table 3.5.

After 24 h of incubation, the fermented BM1 had the highest inhibitory (P<0.05) activity against *B. cereus* and *P. aeruginosa* in comparison to the other fermented BM. No significant differences (P>0.05) found between Both BM1 and BM2 against *E. coli* and *S. aureus*.

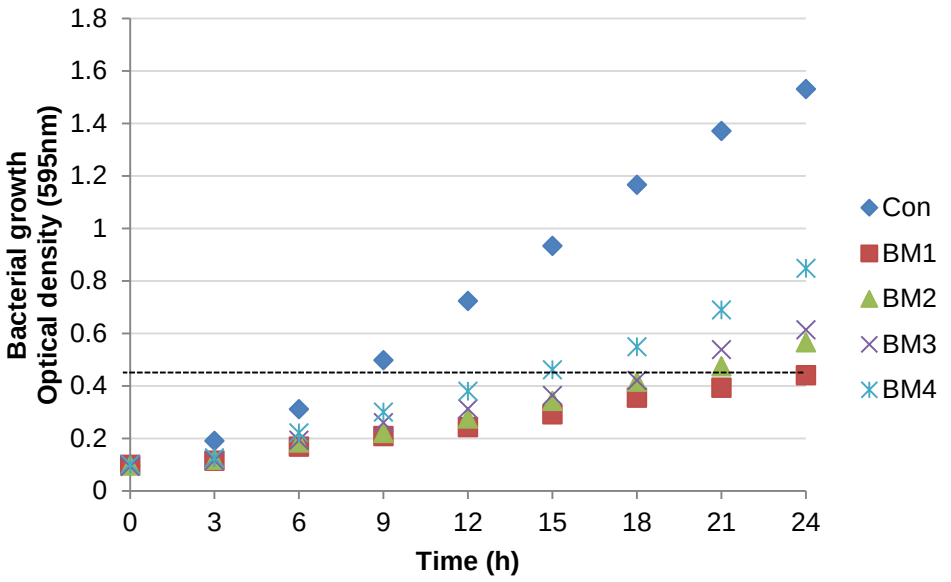
Figure 3.2 shows the growth curves of bacterial strains in BHI broth supplemented with fermented BM and different concentration of nisin every 3h of incubation by using micro-plate reader. The growth curves show that each fermented BM had different inhibitory activity against food pathogenic bacteria, except *B. cereus* and *S. aureus* from both BM1 and BM2, which they were grown at the same range over 24h. During the time over 24h, the growth of food pathogenic strains significantly decreased when the concentration of nisin was increased. Control of pathogen growth is probably due to the activity of metabolites produced by *Lc. lactis* during fermentation of buttermilk and also adding nisin-produced by *Lc. lactis* directly in different concentration which they have antimicrobial activity against food pathogenic and spoilage microorganisms. This study found that the BM1 had an inhibitory activity equivalent to 9µg/ml of nisin after 24h of incubation (Figure 3.2).

Table 3.5: Microbial growth* (OD_{595}) at 24h of incubation at 37°C, as affected by fermented buttermilks' supernatant or concentration of nisin

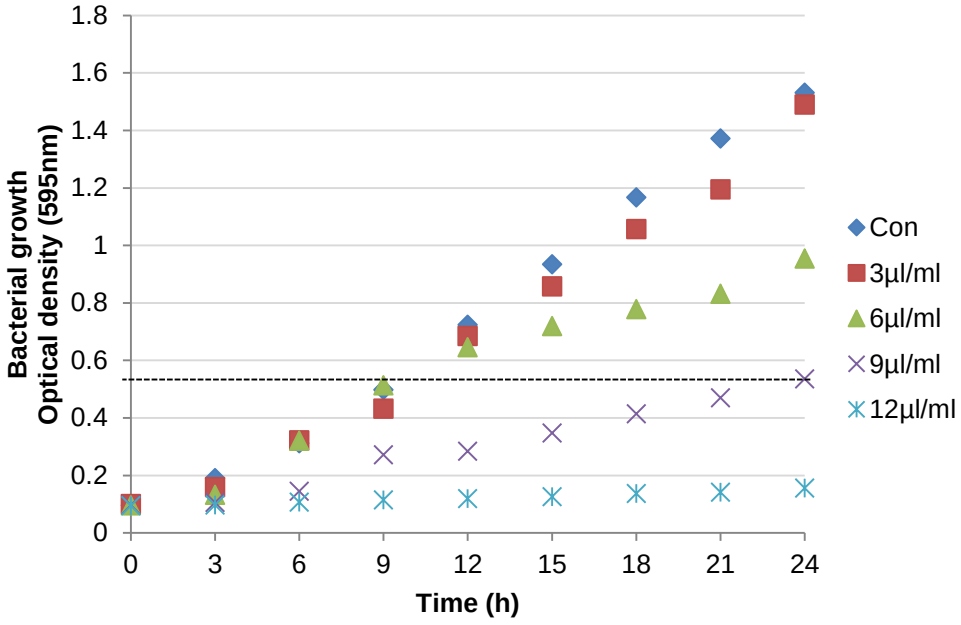
Antimicrobial Component	BM type**	Con. (µg/ml)	Target strains			
			B. cereus	*E. coli*	*P. aeruginosa*	*S. aureus*
Control		0	1.53±0.06 ^{a}	1.35±0.04 ^{a}	1.59±0.04 ^{a}	1.48±0.07 ^{a}
Fermented buttermilk (1%)	BM1		0.44±0.04 ^{d}	0.55±0.03 ^{e}	0.47±0.02 ^{e}	0.45±0.02 ^{ef}
	BM2		0.56±0.02 ^{c}	0.58±0.02 ^{e}	0.59±0.02 ^{d}	0.53±0.01 ^{e}
	BM3		0.61±0.03 ^{c}	0.72±0.03 ^{d}	0.64±0.02 ^{d}	0.82±0.02 ^{c}
	BM4		0.84±0.03 ^{b}	1.09±0.01 ^{c}	0.78±0.02 ^{c}	1.21±0.03 ^{b}
Nisin suspension		3	1.48±0.05 ^{a}	1.21±0.04 ^{b}	1.26±0.03 ^{b}	1.17±0.03 ^{b}
		6	0.95±0.02 ^{b}	0.74±0.05 ^{d}	0.78±0.03 ^{c}	0.63±0.04 ^{d}
		9	0.53±0.03 ^{cd}	0.58±0.04 ^{e}	0.48±0.04 ^{e}	0.38±0.01 ^{f}
		12	0.15±0.01 ^{e}	0.22±0.02 ^{f}	0.19±0.01 ^{f}	0.14±0.01 ^{g}
			R^2 = 0.96	R^2 = 0.97	R^2 = 0.99	R^2 = 0.98

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-g}$ Means in the same column with different superscripts are significantly different (P<0.05). ** Key of the table as Table 3.2

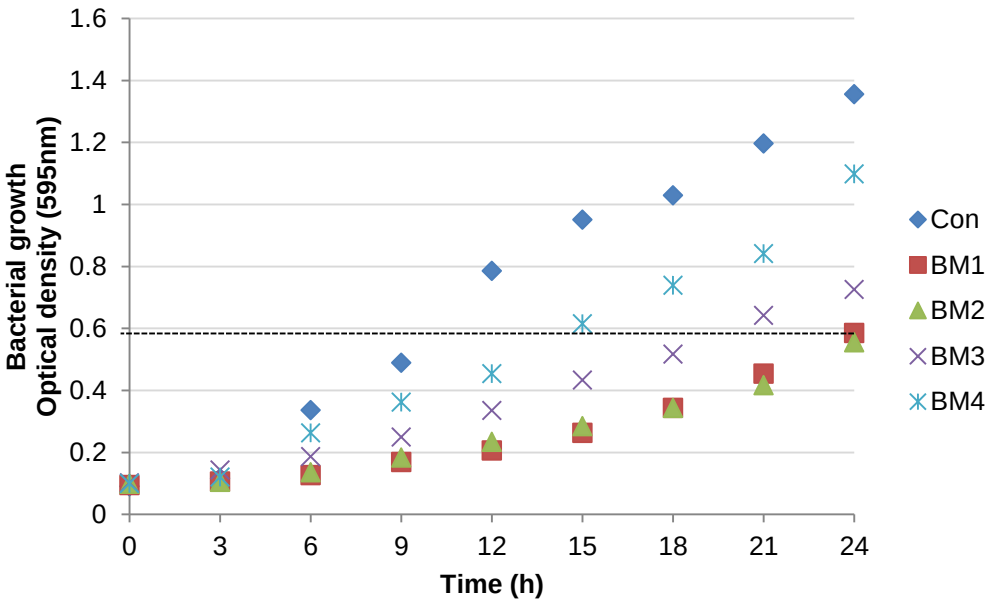
(a) *B. cereus* with fermented buttermilks



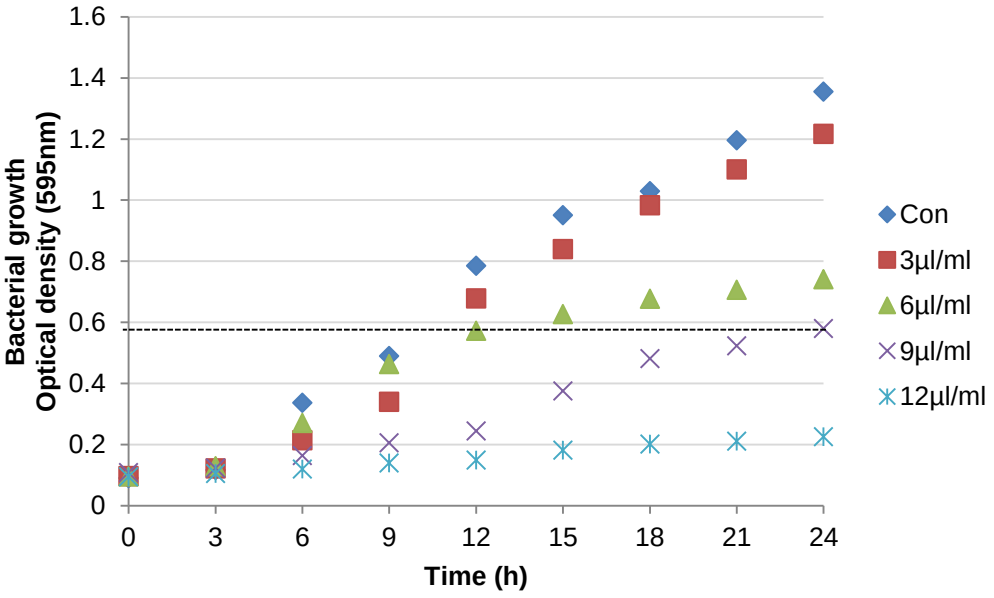
(b) *B. cereus* with different concentrations of nisin



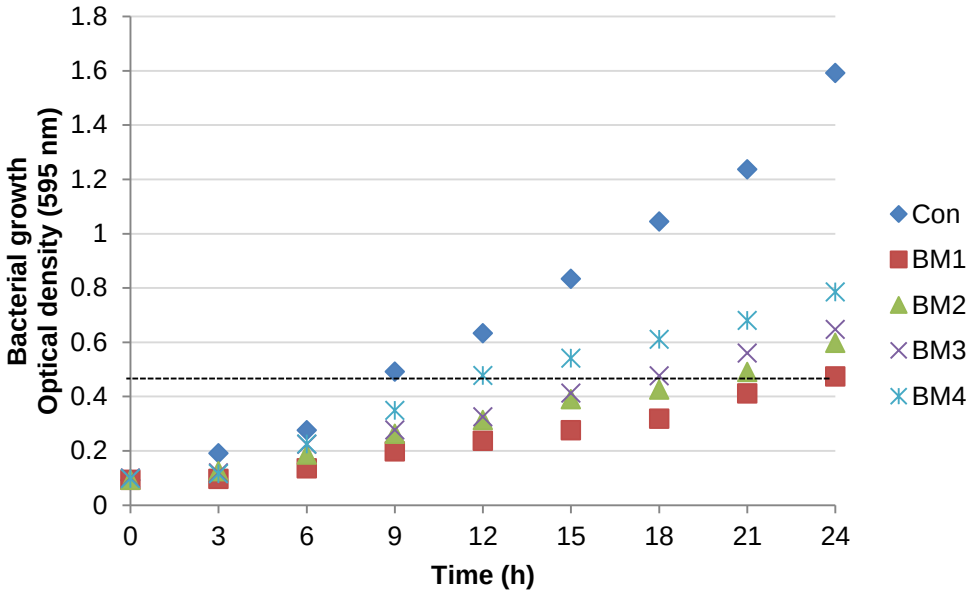
(c) *E. coli* with fermented buttermilks



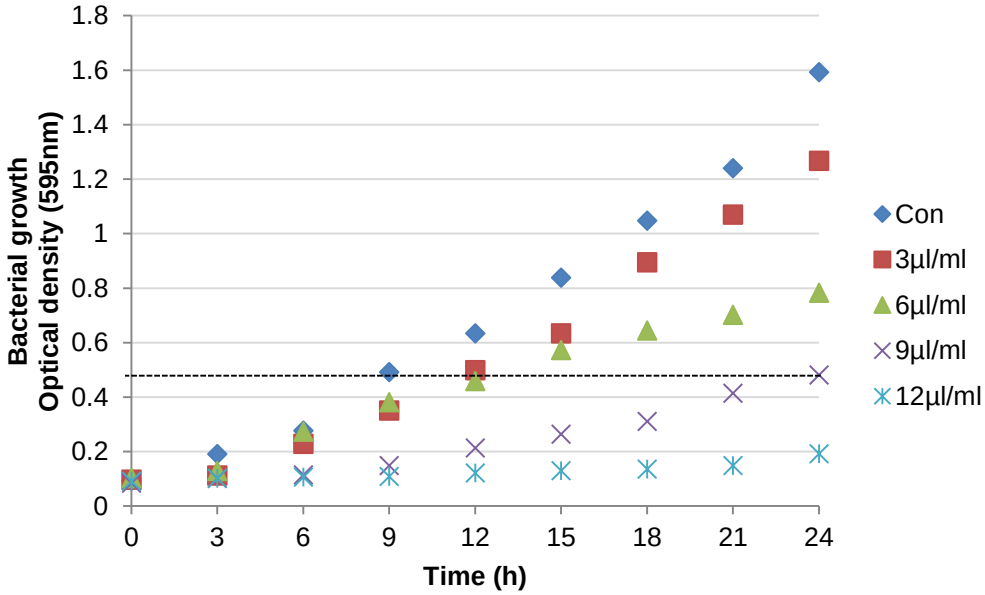
(d) *E. coli* with different concentrations of nisin



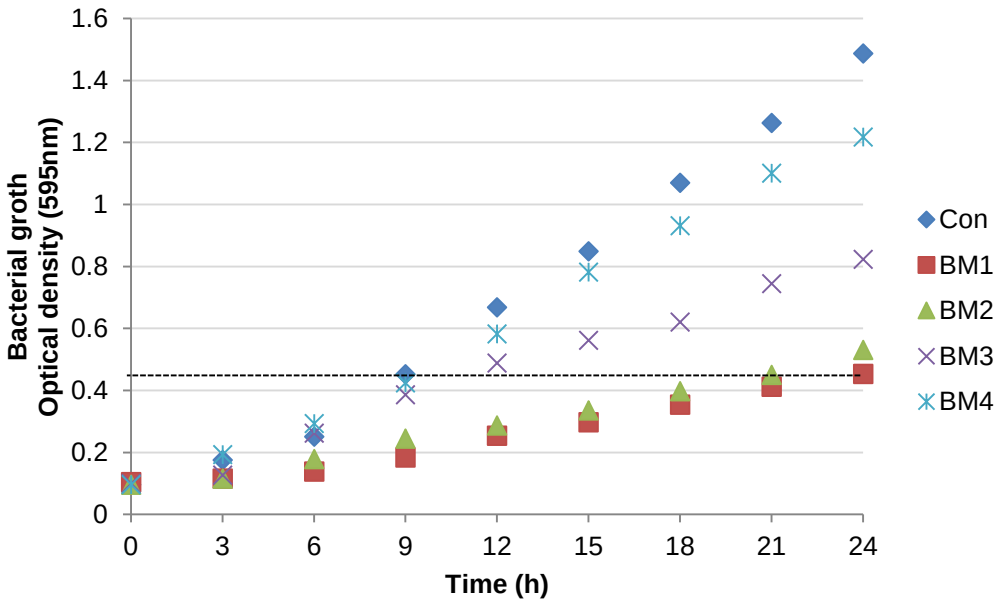
(e) *P. aeruginosa* with fermented buttermilks



(f) *P. aeruginosa* with different concentrations of nisin



(g) *S. aureus* with fermented buttermilks



(h) *S. aureus* with different concentrations of nisin

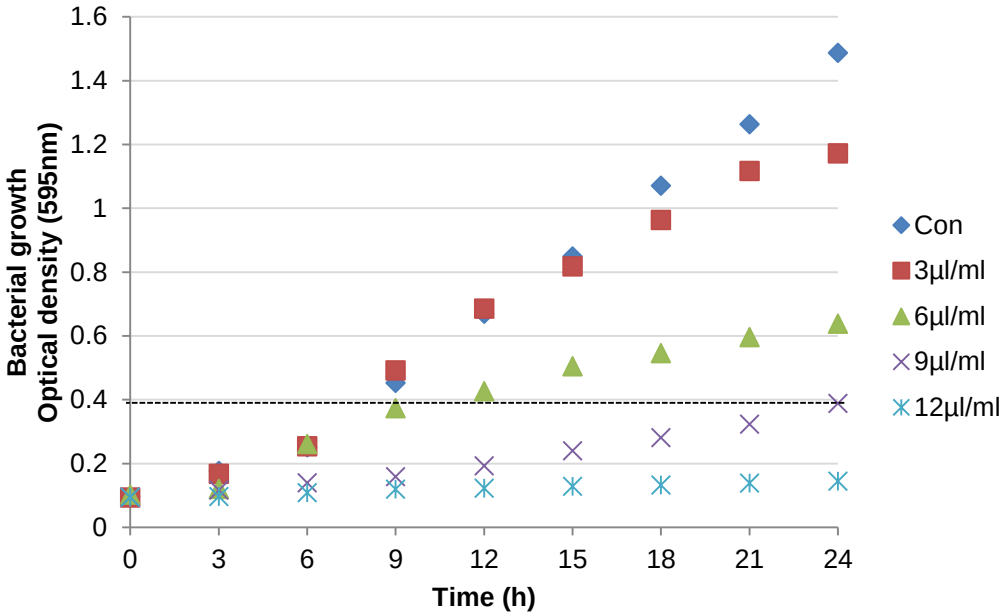


Figure 3.2: Growth curves of target bacterial strains in BHI broth supplemented with supernatant of fermented buttermilk with *Lc. lactis* subsp. *lactis* and different concentrations of nisin every three hours by using micro-plate reader. Results are the means from three replicates

3.3.4 Detection of inhibitory activity of fermented buttermilk supernatant and nisin activity against spores of *Bacillus cereus*

Fermented buttermilk supernatants and different concentrations of nisin were influenced on spores of *Bacillus cereus* as presented in Table 3.6. The *Bacillus cereus* spores were sensitive to all fermented buttermilk supernatants from dilutions 10^{-3} spores/ml. In addition, it was found to be sensitive to fermented BM1 supernatant from dilutions 10^{-1} spores/ml. The *Bacillus cereus* spores were sensitive to fermented BM2 from dilutions 10^{-2} spores/ml. The spores of *Bacillus cereus* were also found to be sensitive to all nisin at the levels tested from dilution 10^{-4} spores/ml, with nisin concentration at 6µg/ml from 10^{-2} spores/ml and from dilution 10^{-1} spores/ml with nisin concentration at (9 and 12)µg/ml.

Table 3.6: *Bacillus cereus* spores sensitivity* to different concentrations of nisin and fermented buttermilk at 37°C for 7 days

number of spores	Control	Nisin (µg/ml)				Fermented buttermilk (1%)			
	0	3	6	9	12	BM1	BM2	BM3	BM4
10^{-1}	+	+	+	-	-	-	+	+	+
10^{-2}	+	+	-	-	-	-	-	+	+
10^{-3}	+	+	-	-	-	-	-	-	-
10^{-4}	+	-	-	-	-	-	-	-	-
10^{-5}	+	-	-	-	-	-	-	-	-

* (+) growth of *Bacillus cereus* after spore incubation at 37°C for 7days, (-): no growth

3.3.5 Antimicrobial activity of nisin and chemical preservatives

The effectiveness of antimicrobial activity of nisin comparing to chemical preservatives for the inhibition of food pathogenic strains were assessed in this study using 96-well micro plate to determine the microbial growth of food pathogenic stains. The microbial growth (OD_{595}) of the four food pathogenic bacteria strains in BHI broth media containing different concentration of commercial nisin (3, 6, 9, 12µg/ml) as reference and potassium sorbate, calcium propionate and vinegar were added into BHI broth 100, 300, 600 and 900ppm with control are shown in Table 3.7.

The growth of food pathogenic bacteria strains (*B. cereus*, *E. coli*, *P. aeruginosa* and *S. aureus*) were decreased significantly ($P<0.05$) by increasing the concentration of nisin and chemical preservatives. 12µg/ml of nisin and 900ppm of calcium propionate had the highest inhibitory activity against the growth of all food pathogenic bacteria strains. 900ppm of both potassium sorbate and vinegar with 600ppm of calcium propionate had inhibitory activities equivalent to 9µg/ml of nisin at all food pathogenic strains.

Table 3.7: Microbial growth* (OD_{595}) at 24h of incubation at 37°C, as affected by chemical preservatives or concentrations of nisin

Antimicrobial component	Con.		Target strains			
	(µg/ml)	(ppm)	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. aureus*
Control	--		1.53±0.06 ^{a}	1.35±0.04 ^{a}	1.59±0.04 ^{a}	1.48±0.07 ^{a}
Nisin	3		1.48±0.05 ^{a}	1.21±0.04 ^{b}	1.26±0.03 ^{bc}	1.17±0.03 ^{bc}
	6		0.95±0.02 ^{d}	0.74±0.05 ^{f}	0.78±0.03d ^{f}	0.63±0.04 ^{g}
	9		0.53±0.03 ^{f}	0.58±0.04 ^{g}	0.48±0.04 ^{h}	0.38±0.01 ^{h}
	12		0.15±0.01 ^{g}	0.22±0.02 ^{h}	0.19±0.01 ^{i}	0.14±0.01 ^{i}
Potassium sorbate		100	1.36±0.03 ^{b}	1.19±0.03 ^{b}	1.33±0.02 ^{b}	1.25±0.03 ^{b}
		300	0.99±0.03 ^{d}	0.93±0.01 ^{d}	0.94±0.02 ^{e}	1.07±0.03 ^{cd}
		600	0.75±0.02 ^{e}	0.73±0.01 ^{f}	0.66±0.01 ^{g}	0.75±0.02 ^{f}
		900	0.52±0.01 ^{f}	0.54±0.02 ^{g}	0.45±0.02 ^{h}	0.41±0.02 ^{h}
Calcium propionate		100	1.14±0.02 ^{c}	1.21±0.02 ^{b}	1.19±0.02 ^{cd}	1.24±0.02 ^{b}
		300	0.84±0.01 ^{e}	0.86±0.01 ^{de}	0.72±0.01 ^{fg}	0.88±0.01 ^{e}
		600	0.51±0.01 ^{f}	0.54±0.01 ^{g}	0.43±0.02 ^{h}	0.42±0.03 ^{h}
		900	0.14±0.01 ^{g}	0.19±0.01 ^{h}	0.14±0.01 ^{i}	0.16±0.01 ^{i}
Malt vinegar 5% (v/v)		100	1.49±0.02 ^{a}	1.29±0.02 ^{ab}	1.51±0.02 ^{a}	1.39±0.04 ^{a}
		300	1.09±0.03 ^{c}	1.04±0.04 ^{c}	1.14±0.02 ^{d}	1.05±0.04 ^{d}
		600	0.78±0.02 ^{e}	0.78±0.04 ^{ef}	0.71±0.02 ^{fg}	0.83±0.02 ^{ef}
		900	0.49±0.04 ^{f}	0.55±0.02 ^{g}	0.44±0.03 ^{h}	0.47±0.03 ^{h}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-i}$ Means in the same column with different superscripts are significantly different (P<0.05).

3.4 Discussion

Three different BM prepared by three different methods and a frozen BM were fermented with *Lc. lactis* subsp. *lactis* and as well as different concentrations of nisin (3, 6, 9, 12 µg/ml) were used as a reference. They were used to evaluate their effectiveness on food pathogenic bacteria using *in-vitro* method. The aim of this chapter is to select the best BM fermented with *Lc. lactis* subsp. *lactis,* with a high inhibitory activity against spoilage and pathogenic microorganisms, which they might be found in bakery products e.g. crumpet bread. Then it could be suitable to be added to the bread crumpet formulations for increasing the quality, texture and shelf life of bread crumpet.

The present study showed that the four different types and preparation method of BM products with *Lc. lactis* subsp. *lactis* had significantly different values of pH and TA values. The pH level of all types and preparation methods of buttermilk ranged between 6.67 and 6.69 before incubation period of buttermilk products. The rate of fall in the pH level of fermented BM was significantly different according to the type of buttermilk products and preparation methods. After 24h incubation period, the pH levels between fermented buttermilks of up to 4.92 to 5.39. BM1 with *Lc. lactis* subsp. *lactis* had the lowest pH value and highest TA value, which might be due to the conversion of sugars in the BM through fermentation to organic acids at different levels as the *Lc. lactis* subsp. *lactis* is homo-fermentative. It can produce more than 95% lactic acids from lactose fermentation which is due to decrease pH and increase acidity of food (Hutkins, 2006). The result was similar with findings of Sodini *et al*. (2006) who reported that different types of buttermilk products have different pH values, which it is around 5.39-6.61. *Lc. lactis* is one of the most important

microorganisms in the dairy industry, because of the capability to produce lactic acid from lactose and drop down in pH level which have an important role in inhibiting growth of microorganisms (Cabo *et al*., 2002; Walker and Klaenhammer, 2003).

Comparing the fermented BM products with different concentration of preservative nisin, fermented BM1 had an inhibitory activity equivalent to 9µg/ml of nisin against food pathogenic strains. Fermented BM1 with *Lc. lactis* subsp. *lactis* could be a suitable candidate to add to crumpets formulation.

In comparison to other fermented BM products, highest inhibitory activity was observed in fermented BM1 with *Lc. lactis* subsp. *lactis* against food pathogenic strains such as *B. cereus*, *E. coli*, *P. aeruginosa* and *S. aureus*. The level of inhibitory activity of fermented buttermilk products might be due to antimicrobial substances which they produced by the *Lc. lactis* subsp. *lactis* through fermentation. The researcher reported that the antagonistic effect could be due to organic acids and antimicrobial substances such as hydrogen peroxide, diacetyl, bacteriocins and low-molecular-weight metabolites which produced by LAB that inhibit pathogenic organisms (Oluwafemi and Adetunji, 2011). Our findings of the results of fermented BM1 products with *Lc. lactis* are in agreement with the results of Millette *et al*. (2004) where they found *Lc. lactis* subsp. *lactis* inhibiting the growth of *E. coli* and *Staph. aureus*. There were no or weak (6–9mm) diameter zone against pathogenic strains which might be due to loss or reduction of bacteriocin activity which it has been widely reported in food matrices and may be caused by a host of factors, including food constituents, pH and proteases (Gálvez *et al*., 2007).

In general, antimicrobial activities of commercial preservative nisin, four types of fermented buttermilk products with *Lc. lactis* subsp. *lactis* and chemical preservatives (potassium sorbate, calcium propionate and vinegar) was found to inhibit the growth of the food pathogenic strains depending on the compounds tested and targeted bacteria. Lactic acid has effects on the membranes of bacteria by forming pores on it, which results in inhibition or death of the bacteria (Delves-Broughton, 2005). According to the LAB and their bacteriocins, the results are similar with findings Cizeikiene *et al*. (2013) which they showed that the metabolites of LAB have inhibitory activities against food pathogenic bacteria, belonging to *Bacillus*, *Pseudomonas*, *Escherichia* and *Listeria* genera in various degrees. LAB have an ability to ferment foods and produce several antimicrobial activities which affects food pathogenic bacteria in food products (De Vuyst and Leroy, 2007).

Fermented buttermilk supernatants and different concentrations of nisin had influenced on different levels of spores of *Bacillus cereus*, which it is more sensitive to fermented BM1 and nisin at 9 and 12µg/ml (Table 3.6). The results agreed with Jenson *et al*. (1994) who found the *Bacillus cereus* spores sensitivity to nisin and Delves-Broughton (2005) who demonstrated that nisin-produced from *Lc. lactis* subsp. *lactis* has an antimicrobial activity against Gram-positive bacteria and the spore forming of bacteria. *Lc. lactis* produces acids which rapidly lower the pH and inhibit the development of undesirable microorganisms. The inhibitory activity of fermented BM supernatants against food pathogenic strains may belong to antimicrobial substances which produced by *Lc. lactis* subsp. *lactis* during fermentation. Nisin-producing by *Lc. lactis* was reported as bio-preservative against *Bacillus cereus* in cooked rice and in milk

(Penna *et al*., 2002). Nisin is less effective on Gram-negative bacteria, as the outer membrane disables the entry of this molecule to the site of action (Boziaris and Adams, 2001; Lee *et al*., 2003). The differential antimicrobial activity of fermented BM supernatants and pure nisin may belong to un-purified fermented BM supernatants. Cintas *et al*. (1998) observed increase in nisin activity after the purification and related to the removal of inhibitors of bacteriocin activity during the purification and/or to a conformational change of the molecule to a more active form in the hydrophobic solvent.

The addition of chemical preservatives and nisin additive was found to have inhibitory activities on all food pathogenic strains, which is concentration dependent. This result is similar to those from previous studies that observed the effect of chemical preservatives in inhibiting the growth of foodborne pathogenic bacteria, also with increasing the concentrate of chemical preservatives due to decrease in the growth of pathogenic bacteria (Pranoto *et al*., 2005; Oladapo and Abiodun, 2014).

Further studies are needed for gathering the knowledge about the application of fermented BM with starter culture *Lc. lactis* and its bacteriocin as an intermediate ingredient for making bread products. This is to understand the effect of the fermented BM directly on the bread product including the quality changes and shelf life by preventing the growth of undesirable microorganisms.

3.5 Conclusion

This study revealed that BM products fermented with *Lactococcus lactis* subsp. *lactis* and commercial nisin (3, 6, 9, 12µg/ml) has different activities on some food pathogenic bacteria using *in-vitro* method. The lowest value of pH and the highest TA value in the experimental preparation in this study were found in the fermented BM1, which might be due to the conversion of sugars in the BM through fermentation to organic acids at different levels as the *Lc. lactis* subsp. *lactis* is homo-fermentative. Levels of 9 µg/ml of nisin had an inhibitory activity against some food pathogenic bacteria equivalent to 900 ppm of both potassium sorbate and vinegar with 600ppm of calcium propionate which they are used as preservatives in bread production. Additionally, the fermented BM1 and nisin at 9 and 12µg/ml demonstrated the highest inhibitory activity against some food pathogenic bacteria and *Bacillus cereus* spores. Moreover, the fermented BM1 had an inhibitory activity equivalent to 9µg/ml of nisin.

Consequently, further studies are needed to evaluate the effect of adding fermented BM1 and 9µg/ml of nisin directly to the bread crumpet formulations (instead of using chemical preservatives), which might be suitable for increasing the safety, quality, texture, colour changes and extending the shelf life with delaying staling of bread crumpet, this has been demonstrated as discussed in the next chapter.

CHAPTER FOUR

Effects of bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, and fermentation of Buttermilk products on the shelf life and safety of bread crumpets

4.1 Introduction

LAB are the most important bacterial group used in the fermentation of dairy products such as yogurt, cheese, sour milk and butter. Also in fermented meats, and also in combination with yeast are commonly used to ferment cereal products such as sourdoughs (Lavermicocca *et al*., 2000; Ryan *et al*., 2008; Ravyts *et al*., 2012). LAB can be utilised as a starter culture in the bread industry, which increases the sensory properties and prolongs the shelf life and also delay bread staling during storage period of bakery products (Plessas *et al*., 2008). However, the shelf life among breads are different, for example Axel *et al*. (2015) reported that the shelf life of quinoa breads (gluten-free sourdough bread) containing *Lb. amylovorus* fermented sourdough increased for 4 days compared to the non-acidified control. Nowadays, consumers are aware of the health concerns regarding food additives; the health benefits of “natural” and “traditional” foods, processed without any addition of chemical preservatives, are becoming more attractive. One of the alternatives to satisfy this request are bacteriocins, which are antimicrobial peptides produced by a large number of bacteria, including LAB, normally acting against closely related and some spoilage and disease-causing Gram-positive pathogens e.g. *Bacillus* sp., *Clostridium* sp. and *Staphylococcus aureus* (Balciunas *et al*., 2013). For this reason they are used in several applications, among which are biopreservation, shelf-life extension, clinical antimicrobial action and control of fermentation

microflora (Balciunas *et al*., 2013). Parada *et al*. (2007) reported that bio-preservation systems in food products are of increasing interest by the manufacturer and the consumers in recent years.

Bread is one of the most essential products of wheat flour in the world especially in the developing countries (Plessas *et al*., 2008). Crumpets are an unsweetened bakery good, popular in the United Kingdom, Australia and New Zealand. Commercially crumpets properties are non-acid pH (pH 6-9), high moisture (48–56%) and high water activity (0.95–0.99) (Delves-Broughton, 2005; Koukoutsis *et al*., 2005). In general, the product is sold at ambient temperature and has a shelf life of five days and it is changed based on preservatives use. Because of these properties, crumpets have been involved in food poisoning due to growth and toxin production by *Bacillus cereus* during storage at ambient temperature (Jenson *et al*., 1994). *Bacillus cereus* produces one emetic toxin (intoxication) and three different enterotoxins (diarrhoeal infection) (Granum and Lund, 1997). The intoxication is occurred in rice cooked for a time and temperature insufficient and when is improperly stored in order to kill any spores present. It can produce a toxin cereulide if the vegetative cell count exceeds 10^5 CFU/g, which is not inactivated by later reheating. This form leads to nausea and vomiting 1-5h after consumption (Watson, 1998). A diarrhoeal infection is due to the ingestion of bacterial cells which produce enterotoxin in the small intestine. This infection occurs when *Bacillus cereus* levels exceed 10^6 CFU/g in the food and sufficient amounts of the enterotoxin are formed in the small intestine of the host. Two of the three enterotoxins are proved to be involved in food poisoning. Both of them consist of three different proteins that interact.

This form leads to abdominal pain, diarrhoea and nausea 8-16h after consumption (Granum, 1994; Granum and Lund, 1997).

The Regulation (EC) No. 2073/2005 differentiates the general microbiological criteria for the food safety hygiene and the process hygiene which indicates whether or not the production process is operating in a hygienic manner. They are applicable to foodstuffs placed on the market during their shelf life. Although European regulations do not provide specific criteria for *Bacillus cereus*, the Regulation (EC) No. 1441/2007 introduced a requirement for presumptive *Bacillus cereus* in dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age. This criterion is applicable at the end of manufacturing.

The main component of crumpet formulations is flour which may invariably contain low numbers of *Bacillus cereus* spores. Baking kills vegetative micro-organisms and mould spores, but cannot destroy bacterial spores. After the hot plate cooking and during the 3–5 days when the crumpets are stored at ambient temperature, cells could grow to levels of public health concern (10^5 CFU/g) which can be sufficient to cause food poisoning (Jenson *et al*., 1994; Smith *et al*., 2002) by toxin production, but other health risks are explained in Section 1.9. In some cereal products, and particularly bread made without dough acidification, the combination of the hurdles pH (5.4±6.0) and Aw (0.94±0.97) may still allow growth of some *Bacillus* strains (Zeuthen and Bøgh-Sørensen, 2003). Control of *Bacillus cereus* spores in crumpets can be achieved through low temperature storage and most commonly through the use of chemical preservatives such as sorbic and propionic acids and their salts. Nisin, a bacteriocin produced by *Lc. lactis,* is also permitted as a preservative to control

the growth of *Bacillus cereus* in crumpets. Addition of nisin to the batter mix at 3.75 mg/kg to prevent the growth of *Bacillus cereus* spore has received regulatory approval in Australia and New Zealand (Jenson *et al*., 1994). Also, it is permitted as a food preservative by FSA in the UK. Bread crumpets were used in this study because of the food poisoning outbreak caused by *Bacillus cereus*, to keep them safe for longer shelf life, delay staling and increase the quality by adding BM fermented with *Lc. lactis* subsp. *lactis*. Moreover, preserve bread crumpets by bio-preservatives instead of using chemical preservatives.

The aims of this study were:

- To investigate the potential changes on crumpets texture properties and sensory attributes after treatments with fermented buttermilk product by *Lc. lactis* and natural preservative nisin
- To investigate the effects of buttermilk product fermented by *Lc. lactis* and natural preservative nisin on the colour of bread crumpets
- To assess the ability of natural preservative nisin and fermented buttermilk product to kill or restrict the growth and proliferation of pathogenic and spoilage microorganisms, to improve safety and shelf life of crumpets

4.2 Materials and Methods

4.2.1 Preparation of bread crumpets

The crumpets were prepared as described by Daifas *et al*. (2003) with some modifications. Four types of English-style crumpets were used in the study as follows: and the project of experiment was done as shown in Figure 4.1.

CON = Control group (Standard crumpets without additive).

NIS= Standard crumpets with added natural preservative nisin (9 µg/g of batter).

NFBM= by using buttermilk (non-inoculated crumpets)

FBM= with fermented buttermilk (inoculated crumpets with *Lc. lactis* subsp. *lactis*) at 30ºC for 24h with 10^9 cells/ml.

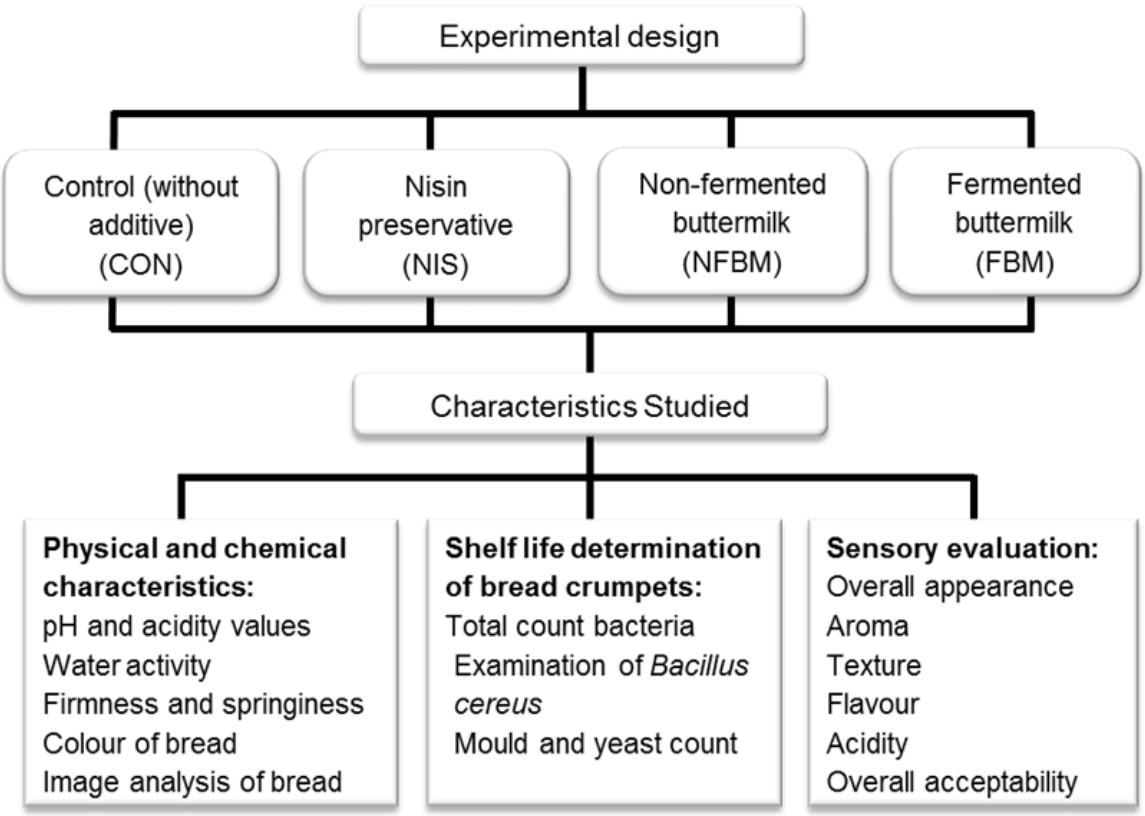


Figure 4.1: The experimental design

4.2.2 Basic ingredients and crumpet Formulation

Bread making: the formulation were made by mixing 450g strong white bread flour, 8g salt, 8g sugar, 12g dried active yeast, 70g skim milk powder, 630g water. Skim milk powder was replaced by the same amount of buttermilk in NFBM and FBM treatments as shown in the Table 4.1.

Table 4.1: Treatment formulations* of bread crumpets (g/100g of mixture)

Ingredients	Treatment**			
	CON	NIS	NFBM	FBM
Skim milk powder	5.94	5.94	-	-
Buttermilk powder (BM1)	-	-	5.94	5.94

* Strong white bread flour (38.2g/100g of the mixture), Salt (0.67g/100g of the mixture), Sugar (0.67g/100g of the mixture), Dried active yeast (1.00g/100g of the mixture), Water (53.5g/100g of the mixture)

** CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

4.2.3 The procedure of making bread crumpet

Skim milk and water were heated to 40°C in a pan for the CON and NIS treatments and skim milk powder was replaced by buttermilk for NFBM and FBM treatments, when the buttermilk in FBM treatment was fermented with *Lc. lactis* subsp. *lactis* for 24h at 30ºC. The liquid were then poured into a bowl and sugar and yeast were added on to the liquid, the mixture were stirred well and left in a warm place for about 10-15min so the mixture started to ferment to get a good frothy head. Flour and salt were added into the mixture and stirred together with a wooden spoon to get a smooth batter (9 µg/g of nisin was added to the batter of NIS treatment and stirred well). The batter were covered and left in a warm place for about one hour until the batter looked creamy and frothy.

For cooking the crumpet, the inner surface of the metal rings and the frying pan were greased over the heat, the rings were arranged in the frying pan. When

the temperature reached around 150ºC, 50g of crumpet batter was poured into each ring, after a while, crumpets started to bubble and formed holes on the surface which indicate that the crumpet is setting. Later, the crumpets were cooked for 5-6min then flipped. After that, the ring and the crumpet were separated and cooked for one more minute. Finally, crumpets were cooled to room temperature and stored in polyethylene bags then kept in room temperature.

4.2.4 Physical and chemical properties of crumpets

4.2.4.1 PH and TA values

The pH of batter before baking, and bread crumpets were measured every two days during storage until they were spoiled as described in Section 2.7.1. To measure the TA, the solutions of bread samples were prepared for pH value and then titrated against 0.1N NaOH with phenolphthalein indicator. TA was expressed as lactic acid percent as described in Section 2.7.2.

4.2.4.2 Water activity measurement for bread samples

Water activity for bread crumpet samples was determined every two days at storage up to spoiled were determined as described in Section 2.7.3.

4.2.4.3 Measuring the colour of bread crumpets

The colour of bread crumb and crust was measured as mentioned in Section 2.7.4.

4.2.4.4 Measuring firmness and springiness of bread crumpets

Bread texture was analysed to assess firmness and springiness (elasticity) using texture analyser (TA-TX2-Stable Micro System, UK) calibrated with a 5Kg weight. Circles of bread (20mm diameter) were taken from the bread and tested. The settings were used as pre-test speed: 1.0mm/s; test speed: 1.0mm/s; post-test speed; 10mm/s; return distance: 5mm; auto 5g trigger force and a 36mm diameter cylindered probe with radius (P/36R) were used. Texture analysis was measured every two days during storage of bread samples. Six replicates were measured for each treatment.

4.2.4.5 Image analysis based measurement of bread porous structure

Bread crust crumpets were photographed with a digital camera (Sony Cyber shot DSC-HX30V, Japan). The images were analysed using the software Image J version 1.49 (Braadbaart and Van Bergen, 2005; Datt *et al*., 2007) that uses the contrast between the two phases (pores and solid part) in the image. The image was firstly converted to grey scale, and then the images were stored in a format of 3256×3225pixels. Using bars of known lengths, pixel values were converted into distance units. After adjusting the threshold, area-based pore size distribution, median pore diameter and pore area as fraction of total area were determined using the software image J. Measurements were performed in triplicate.

4.2.5 Microbial shelf life determination of bread crumpets

Crumpets were taken aseptically for microbiological analysis (aerobic plate count (APC), mould and yeast counts and *Bacillus cereus*) as described in Section 2.7.5.

4.2.6 Sensory evaluation

Samples of crumpets; control (no additives), crumpets with NIS, NFBM and FBM were subjected to sensory evaluation by 33 panellists as described in Section 2.8.

4.2.7 Statistical Analysis

All data were analysed statistically as detailed in Section 2.9.

4.3 Results

4.3.1 pH and TA values of bread crumpets

Bread samples were produced using fermented buttermilk with *Lc. lactis* subsp. *lactis* and compared to samples including non-inoculated buttermilk, natural preservative nisin, and Control. The pH and TA values of bread batter and bread crumpets are shown in Table 4.2. There were significant differences ($P<0.05$) in pH and TA values between treatments. The pH values varied from 5.43 to 5.95 and TA values varied from 0.25 to 0.39mg/100mg for bread batter after 1h fermentation. Whereas the pH values ranged from 5.63 to 6.26 and TA values from 0.19 to 0.33 for bread crumpets after made directly. The pH of bread batter and bread crumpets with FBM was lower than other treatments, while the TA values were higher than the other treatments.

The pH and TA values of bread crumpet samples during storage period at room temperature are shown in Table 4.3. In each time point of storage, the pH values of the bread with FBM was decreased and TA was increased significantly ($p<0.05$) compared to the other treatments. No significant change was found in pH and TA values between bread crumpets with NIS and NFBM over 6 days of storage. There were no significant differences in TA between control and bread with NIS over 6 days of storage. The pH values had decreased significantly ($P<0.05$) for all treatments over 8 days of storage at room temperature. Microbial growth was observed on the surface of the control crumpet bread and crumpets with NFBM on day 8 of storage, so they were discarded and removed from the test.

Table 4.2: pH and TA values* of bread batter and bread crumpets with added natural preservative nisin and fermented buttermilk product

Treatment**	Bread batter		Bread crumpets	
	pH	TA (mg/100mg)	pH	TA (mg/100mg)
CON	5.95±0.02 [a]	0.25±0.02 [a]	6.26±0.01 [a]	0.19±0.01 [a]
NIS	5.91±0.02 [ab]	0.26±0.02 [ab]	6.19±0.01 [b]	0.22±0.02 [ab]
NFBM	5.89±0.005 [b]	0.29±0.01 [b]	6.22±0.01 [ab]	0.24±0.01 [b]
FBM	5.43±0.02 [c]	0.39±0.02 [c]	5.63±0.02 [c]	0.33±0.01[c]

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). [a-c] Means in each column with different superscripts are significant different (P<0.05).

** CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

Table 4.3: Development of pH and TA (mg/100mg) values* of bread crumpets after 0, 2, 4, 6 and 8 days of storage at room temperature

Parameter	Treatment[1]	Time (days)				
		0	2	4	6	8
pH	CON	6.26±0.01 ^{aA}	6.25±0.01 ^{aA}	6.22±0.01 ^{aAB}	6.19±0.01 ^{aB}	Spoiled[2]
	NIS	6.19±0.01 ^{bA}	6.17±0.01 ^{bA}	6.16±0.01 ^{bA}	6.15±0.01 ^{bAB}	6.11±0.01 ^{aB}
	NFBM	6.22±0.01 ^{abA}	6.20±0.01 ^{bAB}	6.27±0.01 ^{bBC}	6.15±0.01 ^{bC}	Spoiled
	FBM	5.63±0.02 ^{cA}	5.63±0.01 ^{cA}	5.61±0.01 ^{cAB}	5.61±0.01 ^{cAB}	5.59±0.01 ^{bB}
TA	CON	0.19±0.01 ^{cA}	0.19±0.01 ^{cA}	0.19±0.01 ^{cA}	0.20±0.01 ^{cA}	Spoiled
(mg/100mg)	NIS	0.22±0.02 ^{bcA}	0.22±0.02 ^{bcA}	0.22±0.01 ^{bcA}	0.22±0.01 ^{bcA}	0.22±0.17 ^{bA}
	NFBM	0.24±0.01 ^{bA}	0.24±0.01 ^{bA}	0.24±0.02 ^{bA}	0.25±0.02 ^{bA}	Spoiled
	FBM	0.33±0.01^{aA}	0.34±0.01 ^{aA}	0.34±0.01 ^{aA}	0.34±0.01 ^{aA}	0.35±0.01 ^{aA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-c}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-C}$ Means in the same row with different superscripts are not significantly different (P<0.05).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] Spoiled: Microbial growth was observed on the surface of the breads

4.3.2 Water acidity of bread crumpets

Changes in the water activity values of bread crumpet samples during storage period at room temperature are shown in Table 4.4. There were significant differences (P<0.05) between the treatments (CON-FBM) during day 0 and 2 of the storage. For the day 4 and 6 of the storage period, there were no significant differences between control and crumpets with NFBM, as well as between crumpets with nisin and crumpets with FBM. The water activity of crumpets with FBM and nisin was significantly lower than the other treatments. Over the storage period, water activity did not change significantly.

Table 4.4: Water activity* of bread crumpets with added nisin and FBM after 0, 2, 4, 6 and 8 days of storage at room temperature

Treatment[1]	Time (days)				
	0	2	4	6	8
CON	0.95±0.00 [a]	0.95±0.00 [a]	0.95±0.00 [a]	0.95±0.00 [a]	Spoiled[2]
NIS	0.93±0.00 [c]	0.93±0.00 [c]	0.93±0.00 [b]	0.93±0.00 [b]	0.93±0.01 [a]
NFBM	0.94±0.00 [b]	0.94±0.00 [b]	0.94±0.01 [a]	0.95±0.01 [a]	Spoiled
FBM	0.92±0.00 [d]	0.92±0.00 [d]	0.92±0.00 [c]	0.92±0.01 [b]	0.92±0.00 [b]

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). [a-d] Means in the same column with different superscripts are significantly different ($P<0.05$). ANOVA followed by Tukey's test with row are not significantly different ($P>0.05$).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] Spoiled: Microbial growth was observed on the surface of the breads

4.3.3 Firmness and springiness of bread crumpets

Changes in the firmness and springiness of bread crumpet samples during the storage period at room temperature are shown in Table 4.5. In each time point of storage, firmness of the bread with FBM was decreased and springiness was increased significantly (p<0.05) comparing to the other treatments. Over 2 days of storage, there were no significant differences in firmness and springiness between crumpets with NIS and NFBM, and no change happened to the firmness and springiness between control and crumpets with NFBM on day 2 of storage. Day 4, there were no significant differences (P>0.05) in firmness between control and crumpets with FBM. Day 6, there were significant differences in firmness between all the treatments. On days 2, 4 and 6, no changes happened to the springiness of the control and crumpets with NFBM. On day 8, firmness and springiness did not change significantly between crumpets with NIS and FBM. Microbial growth was observed on the surface of control and crumpets with NFBM on day 8, so they were discarded and removed from the test. Furthermore, there were significant increases (p<0.05) in the firmness and decrease in the springiness to all treatments at all days of storage except firmness of FBM between day 0 and 2, also springiness of crumpets with nisin and FBM between day 6 and 8, and NFBM between day 4 and 6. The use of a strain of LAB with particular characteristics appears to be a fundamental requisite to delay hardness. The use of FM by *Lc. lactis* may have been responsible for the decreased firmness. Organic acids produced by LAB have proved to have a beneficial effect on texture and staling which affect the protein and starch fractions and reduce the pH that results in an increase in protease and amylase activities of the flour, thus reducing staling.

Table 4.5: Firmness and springiness* of bread crumpets after 0, 2, 4, 6 and 8 days of storage at room temperature

Parameter	Treatment[1]	Time (days)				
		0	2	4	6	8
Firmness (g)	CON	113.64±9.43 ^{aD}	140.57±9.72 ^{aC}	221.26±5.58 ^{aB}	270.86±8.7 ^{aA}	Spoiled[2]
	NIS	79.49±6.9 ^{bcE}	107.53±12.59 ^{bcD}	179.78±13.81 ^{bC}	242.78±5.61 ^{cB}	275.09±7.78 ^{aA}
	NFBM	91.92±8.06 ^{bD}	121.28±18.08 ^{abC}	213.08±9.14 ^{aB}	256.66±3.73 ^{bA}	Spoiled
	FBM	76.79±10.52 ^{cD}	93.19±6.46 ^{cD}	140.74±7.15 ^{cC}	225.29±10.38 ^{dB}	265.27±14.97 ^{aA}
Springiness (%)	CON	53.09±4.38 ^{cA}	42.85±2.88 ^{cB}	27.13±0.69 ^{cC}	22.17±0.69 ^{cD}	Spoiled
	NIS	75.95±6.55 ^{abA}	56.43±6.53 ^{abB}	33.55±2.81 ^{bC}	24.72±0.56 ^{bD}	21.82±0.62 ^{aD}
	NFBM	65.67±5.51 ^{bA}	50.35±7.11 ^{bcB}	28.20±1.19 ^{cC}	23.38±0.34 ^{cC}	Spoiled
	FBM	79.36±10.76 ^{aA}	64.65±4.76 ^{aB}	42.72±2.14 ^{aC}	26.68±1.31 ^{aD}	22.68±1.36 ^{aD}

* Mean values from six replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-d}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-E}$ Means in the same row with different superscripts are not significantly different (P>0.05).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] Spoiled: Microbial growth was observed on the surface of the breads

4.3.4 Colour of bread crumpets

Whiteness crust (top and bottom) and crumb of bread crumpets are shown in Table 4.6. L^* (lightness) a^* (redness) and b^* (yellowness) of crust (top and bottom) and crumb of bread crumpets were measured as shown in Figure 4.2. Colour analysis of the crust top indicated that crumpets with FBM had significant (P<0.05) lower whiteness and lightness comparing to the control. The crumpets with NIS and NFBM had no significant difference (P>0.05) in whiteness and lightness when compared to the control and crumpets with FBM except crumb colour of crumpets with NFBM and FBM. There were no significant differences of crust bottom whiteness and lightness between all the treatments. The whiteness and lightness of the crumb bread with FBM was significantly lower in comparison with the control and crumpets with NFBM. There were no significant differences for whiteness crumb colour among control, crumpets with NIS and NFBM (Table 4.6 Figure 4.2). There were no significant differences of the redness crust (top and bottom) and crumb colour between all the treatments. According to the yellowness, there were no significant differences of crust top colour between all the treatments, whereas the crust bottom of crumpets with FBM had higher yellowness than control. Furthermore, there were no significant differences of crumb colour crumpets with FBM in comparison with the other treatments (Figure 4.3).

Table 4.6: Whiteness crust and crumb* of bread crumpets with added nisin and fermented buttermilk product

Treatment**	Crust colour		Crumb colour
	Top	Bottom	
CON	59.24±4.03 [a]	43.56±3.24	62.65±1.89 [a]
NIS	55.59±3.77 [ab]	42.43±3.80	60.72±1.72 [ab]
NFBM	57.69±2.07 [ab]	42.74±3.42	62.27±1.52 [a]
FBM	54.06±3.81 [b]	42.00±4.16	59.53±2.43 [b]

* Mean values from nine replicates ± standard deviations (ANOVA was followed by Turkey's test). [a, b] Means within a column with different superscripts differ significantly (P<0.05).

** CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

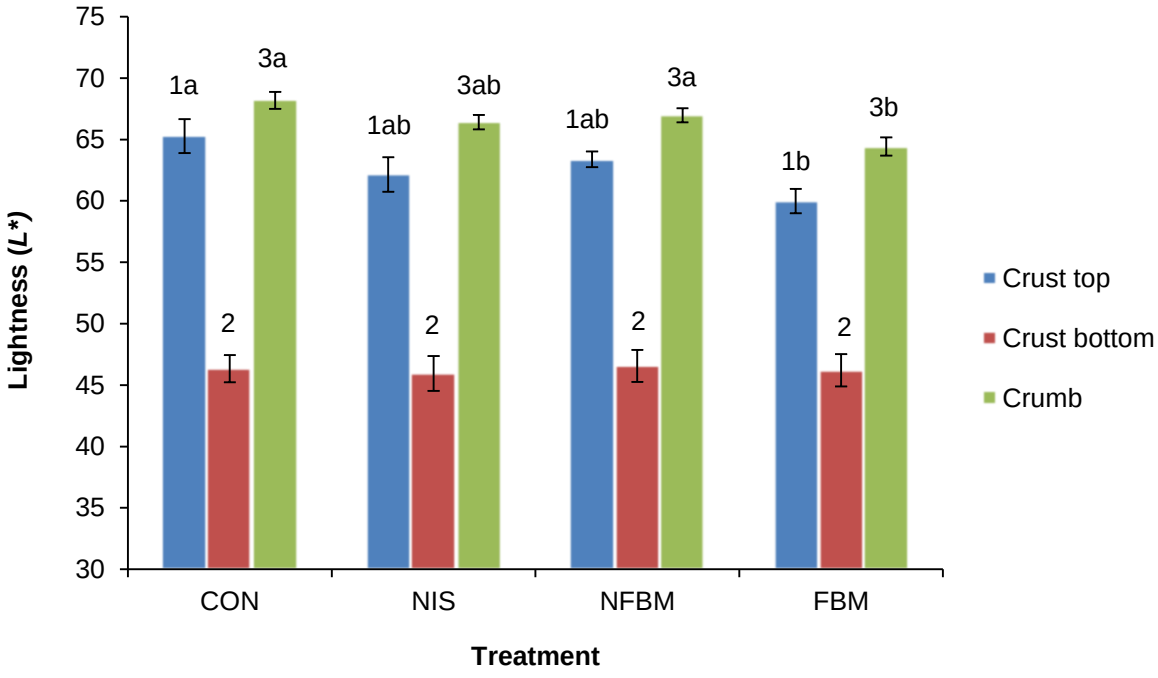
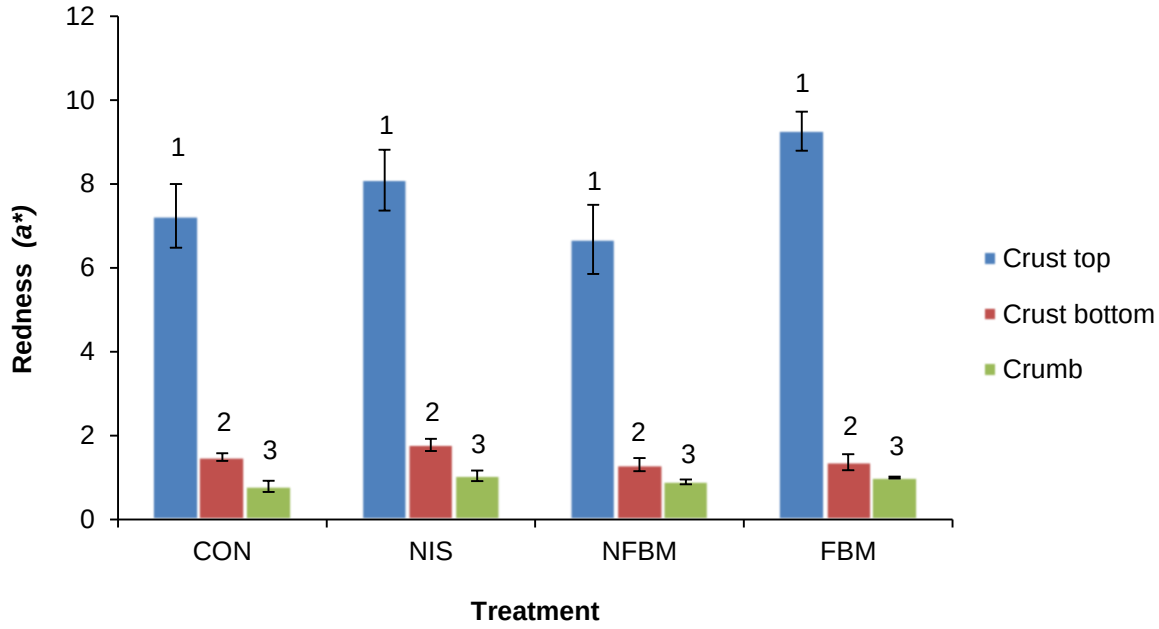


Figure 4.2: Lightness (L) for crust (top, bottom) and crumb of crumpets. Mean values from nine replicates ± standard deviations (ANOVA followed by Turkey's test). [a-b] Within parameter, means with different superscripts are significantly different (P<0.05)

(a*) Redness



(b*) Yellowness

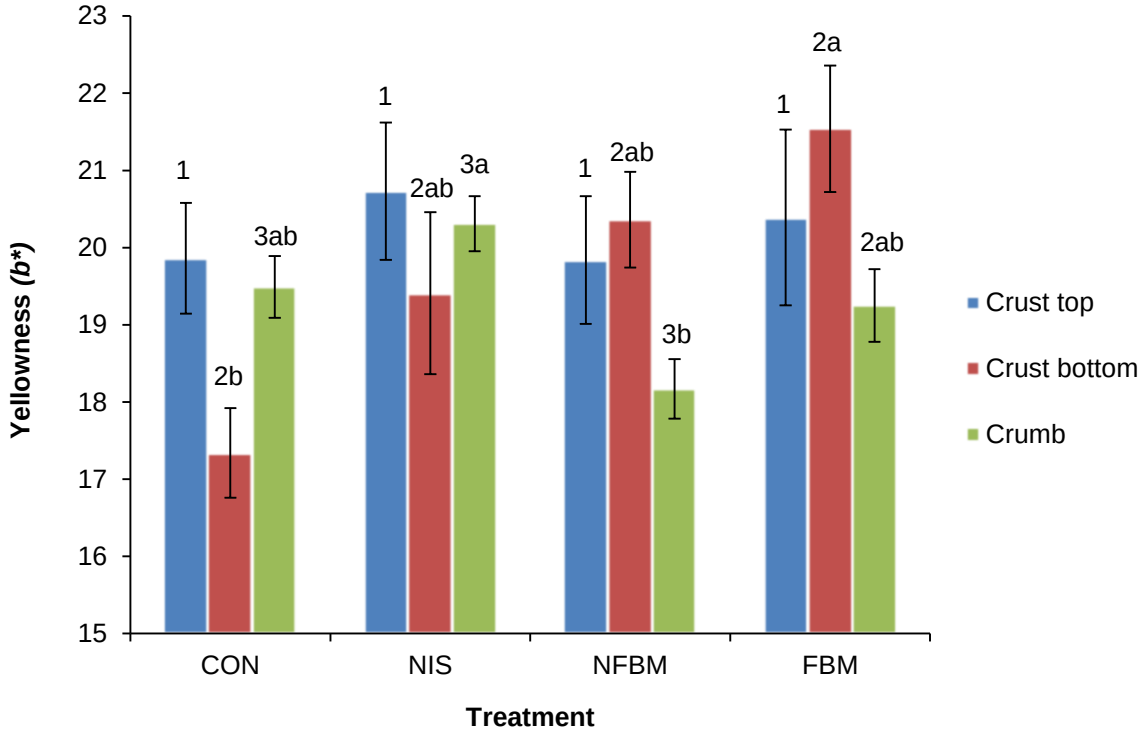


Figure 4.3: Colour (a*) redness and (b*) yellowness values for crust (top and bottom) and crumb of crumpet samples. Mean values from nine replicates ± standard deviations (ANOVA followed by Turkey's test). [a-b] Within same parameter,means with different superscripts are significantly different (P<0.05)

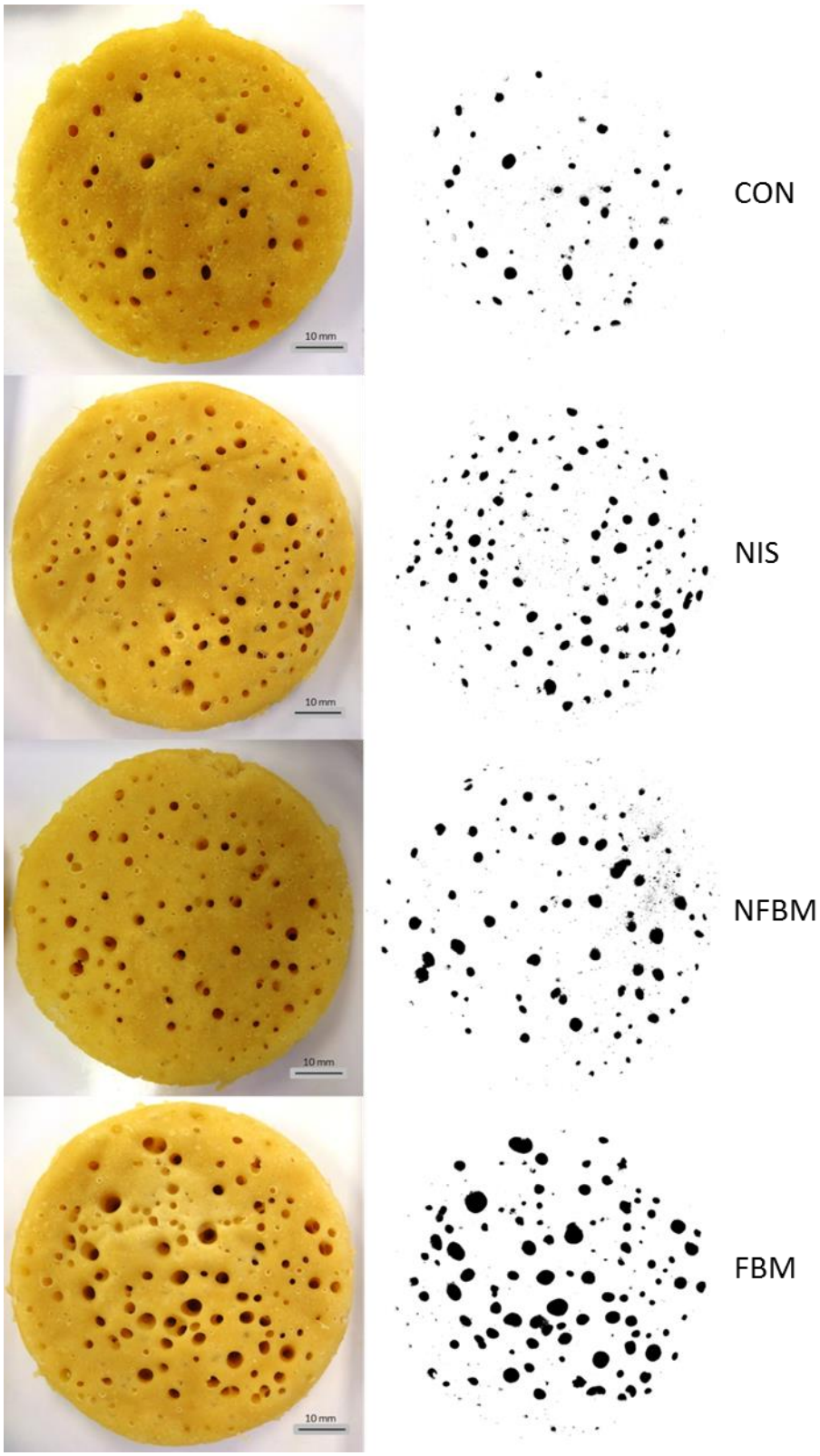


Figure 4.4: Illustration of how the software Image J uses contrast in the image to find the edges of pores and defines the regions representing voids before measuring their areas

4.3.5 Image analysis based measurement of bread porous structure

The photograph images of bread crumpets and pore area of bread crumpets (n=3) are extracted with the image J software to grey scale as shown in Figure 4.5. The pore area distributions obtained for bread crumpets also shown. The percentage of 0.5 mm diameter of control crumpets pore size was higher than the other treatments. From the cumulative data of each treatment, about 42% of the pore size had diameter above 1 mm in control, 53.67 and 56.24% to the crumpets with NIS and NFBM respectively. While about 71.85% of the pore size had diameter above 1 mm in bread crumpets with FBM.

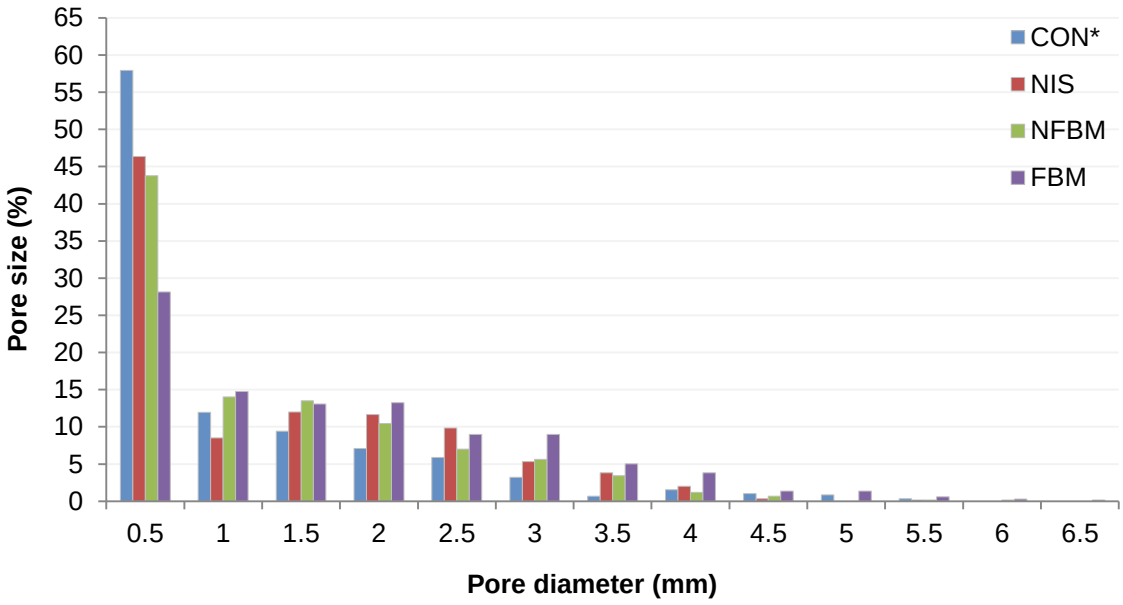


Figure 4.5: Pore size distributions obtained using image analysis of bread crumpets with added nisin and fermented buttermilk product

* CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

4.3.6 Shelf life of bread crumpets

The shelf life of treatment samples was determined as microbial growth during storage at room temperature, shown in Table 4.7, 4.8 and 4.9 between all (CON–FBM). The level of aerobic plate count (APC) was observed in all bread samples (Table 4.7). In all day of storage, the growth of APC in control and crumpets with NFBM was significantly (P<0.05) higher compared to the other treatments. There were no significant differences between control and crumpets with NFBM over spoiled breads except day 2 of storage.

Crumpets with FBM had a significantly (P<0.05) lower APCs in comparison with the other treatments after 8 days of storage at room temperature. APCs increased significantly during storage in all treatments. Microbial growth was observed on the surface of control and crumpets with NFBM on day 8, prompting removal from the test.

The mould and yeast colony counts in bread crumpets over storage period at room temperature are shown in Table 4.8. Moulds and yeasts were not observed in the crumpets with NIS and FBM over 2 days of storage at room temperature. Moulds and yeasts were observed in the control and crumpets with NFBM on day 8 of storage. The number of mould and yeast counts in crumpets with FBM was significantly (P<0.05) lower than the other treatments over 8 days of storage at room temperature. Mould and yeast did not change significantly between control and crumpets with NFBM during storage at room temperature. Over the storage period, moulds and yeasts increased significantly for all treatments.

Table 4.7: Aerobic plate count* (APC) of bread crumpets after 0, 2, 4, 6 and 8 days of storage at room temperature (Log_{10}CFU/g)

Treatment[1]	Time (days)				
	0	2	4	6	8
CON	4.11±0.03 ^{aD}	5.12±0.02 ^{aC}	5.32±0.01 ^{aB}	5.97±0.05 ^{aA}	Spoiled[2]
NIS	3.92±0.02 ^{bE}	4.32±0.01 ^{cD}	4.96±0.03 ^{bC}	5.35±0.01 ^{bAB}	5.83±0.03 ^{aA}
NFBM	4.16±0.03 ^{aD}	5.04±0.02 ^{bC}	5.27±0.01 ^{aB}	5.92±0.05 ^{aA}	Spoiled
FBM	3.81±0.05 ^{cE}	4.27±0.01 ^{dD}	4.83±0.04 ^{cC}	5.05±0.03 ^{cB}	5.73±0.02 ^{bA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-d}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-D}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] Spoiled: Microbial growth was observed on the surface of the breads

Table 4.8: Moulds and yeasts* of bread crumpets after 0, 2, 4, 6 and 8 days of storage at room temperature (Log_{10}CFU/g)

Treatment[1]	Time (days)				
	0	2	4	6	8
CON	n.d.[2]	3.58±0.06 ^{aC}	3.91±0.01 ^{aB}	4.31±0.02 ^{aA}	Spoiled[3]
NIS	n.d.	n.d.	2.91±0.03 ^{bC}	3.57±0.04 ^{bAB}	4.11±0.01 ^{aA}
NFBM	n.d.	3.52±0.03 ^{aC}	3.85±0.01 ^{aB}	4.26±0.02 ^{aA}	Spoiled
FBM	n.d.	n.d.	2.73±0.04 ^{cC}	3.53±0.03 ^{bB}	4.04±0.02 ^{bA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-d}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A, B}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] n.d.: not detected, the detection limit was <10

[3] Spoiled: Microbial growth was observed on the surface of the breads

Table 4.9 shows the growth of *Bacillus cereus* in bread crumpets during storage periods at room temperature. *Bacillus cereus* was not detected in all the treatment until day 6 of storage except control bread and crumpets with NFBM, which were grown on day 6 of storage at room temperature. Crumpets with FBM had a significant (P<0.05) lower number of *Bacillus cereus* in comparison with the other treatments on the day 7 and 8 of storage. *Bacillus cereus* counted significantly in all treatments during storage periods at room temperature. Microbial growth was observed on the surface of the breads, so they were tested. Aerobic colony counts in bakery products is satisfactory at the level of $<10^4$ CFU/g. While, the *Bacillus cereus* is satisfactory at the level of $<10^3$ CFU/g as reported by HPA (2009). The crumpets with FBM and NIS separately had a shelf life of 8 days. While the other treatments had a shelf life of 6 days when tested for the level of APC, mould and yeast colony counts and growth of *Bacillus cereus*. This extend is probably due to the activity of *Lc. lactis* and their metabolites during fermentation of buttermilk and also adding nisin-produced by *Lc. lactis* directly to the crumpets which they have antimicrobial activity against food pathogenic and spoilage microorganisms.

Table 4.9: growth of *Bacillus cereus** of bread crumpets after 6, 7 and 8 days of storage at room temperature (Log_{10}CFU/g)

Treatment[1]	Time (days)		
	6	7	8
CON	2.77±0.05 ^{aA}	3.28±0.03 ^{cB}	Spoiled[3]
NIS	n.d.[2]	2.68±0.04 ^{bA}	2.94±0.03 ^{bB}
NFBM	2.67±0.05 ^{aA}	3.25±0.01 ^{cB}	Spoiled
FBM	n.d.	2.54±0.04 ^{aA}	2.63±0.04 ^{aB}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a\text{-}c}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A, B}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

[2] n.d.: not detected, the detection limit was <10

[3] Spoiled: Microbial growth was observed on the surface of the breads

4.3.7 Sensory evaluation

From the six sensory attributes evaluated, there were no any significant differences (P>0.05) in all attributes such as overall appearance, aroma, texture, flavour, acidity and overall acceptability. The average rank of 33 panel evaluation from each sensory attributes are shown in Figure 4.6 and pairwise comparisons for the sensory attributed was used for comparison between treatments as overall appearance and overall acceptability are shown in Figure 4.7.

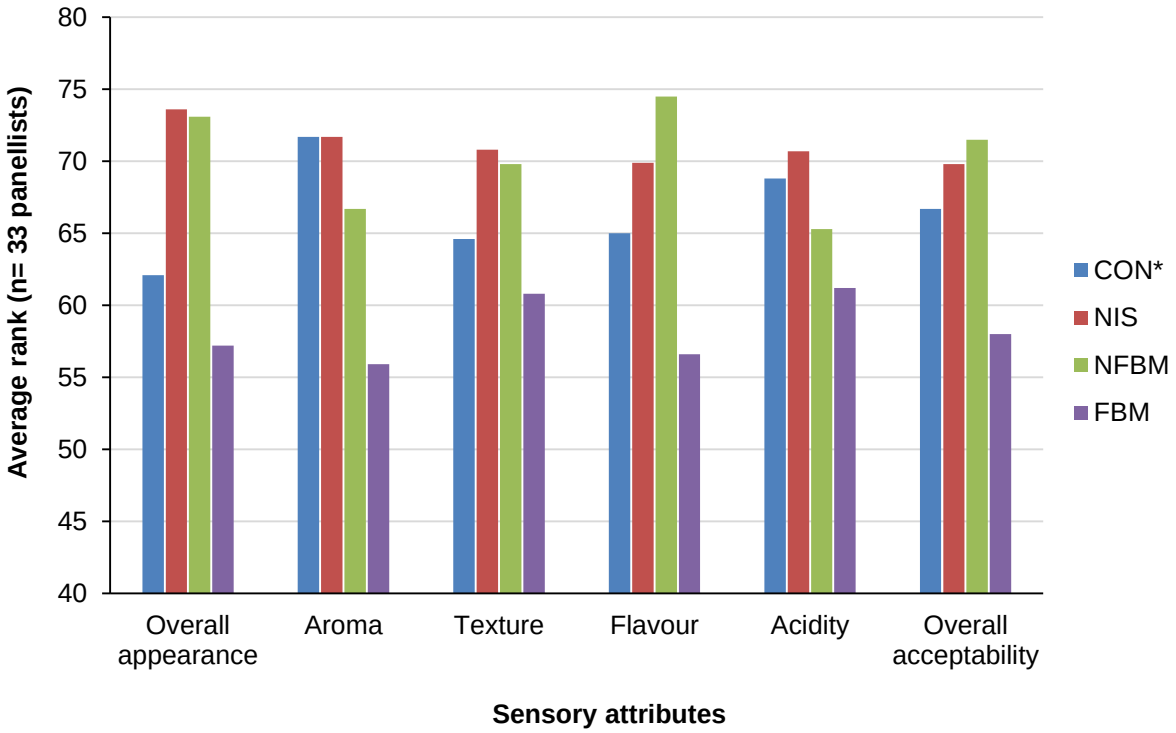


Figure 4.6: Average rank of the sensory evaluation for overall appearance, aroma, texture, flavour, acidity and overall acceptability of bread crumpets with added natural preservative nisin and fermented buttermilk product

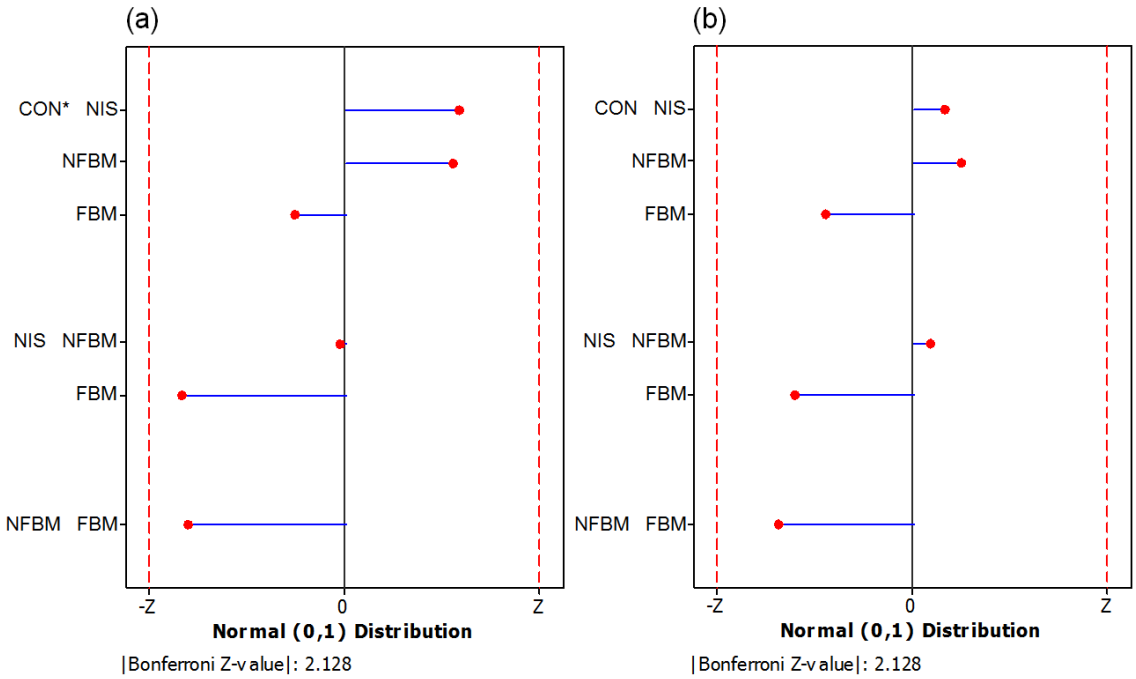


Figure 4.7: Pairwise comparisons of the sensory attributes (a) Overall appearance and (b) Overall acceptability: example of breads using Donn's test

* CON: Control, NIS: Natural preservative nisin, NFBM: Non-fermented buttermilk and FBM: Fermented buttermilk with *Lc. lactis* subsp. *lactis*

4.4 Discussion

The fermented buttermilk with *Lc. lactis* subsp. *lactis* and nisin additive were used to make crumpet breads to improve the bread properties and increase the safety and the shelf life due to reducing growth of pathogenic microorganisms and bacterial sporulation of crumpet breads.

The pH of bread batter and bread crumpets with FBM was lower and TA was higher than the other treatments. Furthermore, the pH value of the bread with FBM was decreased and TA was increased significantly comparing to the other

treatments in each time point of storage. The change in pH level of the batter and bread crumpets with FBM may be due to adding fermented buttermilk with *Lc. lactis* subsp. *lactis* (homo-fermentative LAB) to the batter, which might cause a pH decrease of the batter due to organic acids production (Walker and Klaenhammer, 2003; Hutkins, 2006). It may also increase the shelf life of bread products because of producing antimicrobial substances such as hydrogen peroxide, organic acids, diacetyl, bacteriocins that inhibit pathogenic organisms in fermented foods (Lavermicocca *et al*., 2000; Holzapfel *et al*., 2001). The procedure by which buttermilk was fermented with LAB to decrease pH and produce antimicrobial metabolites was used for making safe and stable crumpets. As an analogy, this was applied by Menteş *et al*. (2007) when instead of fermented buttermilk, they used sourdough fermentation to induce pH decrease. The pH of crumpets for all treatments decreased during the period of storage. This change of the pH may to the growth of some pathogenic microorganisms on the bread crumpets.

The findings of the present study indicate that the firmness of bread crumpets with FBM was softer and springiness was higher in comparison with the control and bread crumpets with NFBM. No significant changes revealed in firmness and springiness between crumpets with NIS and FBM on days 0, 2 and 8 of storage. These results are in agreement with the results of Dal Bello *et al*. (2007) who demonstrated that the crumb firmness of bread fermented by LAB is softer than non-acidifed bread products. Further studies by Clarke *et al*. (2002) and Corsetti *et al*., (2000) have shown that the breads made by adding fermented sourdough instead fermented buttermilk with LAB decreases the crumb firmness values. The different metabolites produced by LAB have proved to

have a beneficial effect on texture and staling. Organic acids affect the protein and starch fractions and reduce the pH that results in an increase in protease and amylase activities of the flour, thus reducing staling (Fadda *et al*., 2014). The pores percent and pores dimensional range of the crumpet breads that inoculated by fermented buttermilk with *Lc. lactis* subsp. *lactis* were higher than the other treatments that are related to the fermentation process and producing metabolites that affected on the crumpet breads' pores. For the water activity, the results were not agreed with results of Dal Bello *et al*. (2007) who reported no significant differences between breads with regard to water activity.

The crust top colour of the crumpets with FBM had significant (P<0.05) lower lightness and whiteness in comparison with the control. There were no significant differences on lightness and whiteness of crumb colour between crumpets with NIS additive, crumpets with NFBM and the control. The results are in agreement with Chiavaro *et al*. (2008) who reported that the bread with adding fermented sourdough had lower lightness on crust colour with no significant differences in the crumb colour. Changes in the crust and crumb colour of the crumpet bread samples may be related to the production of different compounds during the fermentation process.

Rizzello *et al*. (2011) reported that the shelf life of bread can be extended when adding certain LAB strains to bread formulations. The present study showed the shelf life of crumpet breads using FBM and NIS comparing to the control and NFBM (Table 4.7-4.9). The bread crumpets with FBM and crumpets with NIS had a shelf life of 8 days, whereas the control and crumpets with NFBM had a shelf life of 6 days when tested for the level of APC, mould and yeast colony counts and growth of *Bacillus cereus*. The reason is probably due to

metabolites of *Lc. lactis* during fermentation or adding nisin-produced by *Lc. lactis* to the food products which they have antimicrobial activity against food pathogenic and/or spoilage microorganisms as it is presented by researchers (Messens and De Vuyst, 2002; Cooksey, 2005; Sivarooban *et al*., 2007). Based on available information, nisin-produced by *Lc. lactis* has had practical application as a preservative in food processing (Hansen and Sandine, 1994). In the same case, the similar results found from Ogunbanwo *et al*. (2008) who reported that the bread produced with addition of chemical preservative using yeast as starter culture had shelf life of 8 days, however, the combination of *Lb. plantarum* and *Saccharomyces cerevisae* extended the shelf life of bread up to 12 days. The antimicrobial effect of nisin has been demonstrated in crumpets (Jenson *et al*., 1994), 50IU/ml of nisin was added as nisaplin which inhibited 10^1-10^3 spores/g of five *Bacillus cereus* strains. The result was similar with findings of Kikelomo (2012) who reported that the lowest growth of APC and total fungal counts was observed in white layer cake with added commercial nisin in comparison with control sample and cake with added chemical preservatives. LAB have an ability to produce antimicrobial which can contribute in a number of ways towards improving the quality of fermented foods, for instance, through the control of pathogens, prolonging shelf life and improving sensory qualities of food products (Lavermicocca *et al*., 2000; Gerez *et al*., 2008).

Sensory evaluation is one of the most common and useful measurement to assess the quality and acceptability of food product (Elia, 2011). As the results of sensory evaluation have shown there were no significant differences of sensory attributes including overall appearance, Aroma, texture, flavour, acidity,

and overall acceptability between all the treatments. Whereas in previous studies, researchers have reported that LAB and their metabolites are responsible for the characteristic sensory qualities of bread (Beuchat, 1997; Caplice and Fitzgerald, 1999). Finally, it can be concluded that panellists accepted all crumpets. Whereas, there is a considerable difference between sensory attributes. For overall appearance, texture, flavour and overall acceptability crumpets with NIS and NFBM recorded the highest average rank.

4.5 Conclusion

In conclusion, the results of this study demonstrate that the fermented BM with *Lc. lactis* subsp. *lactis* and commercial nisin products had an influence on quality, delay staling and shelf life of bread crumpets product. The bread crumpets with fermented BM showed lower pH values, higher TA values, lower water activity, lower firmness and higher springiness and it effects on the pore size of the crumpets comparing to the other treatments. Consequently, the bread samples with fermented BM and nisin separately had a microbial shelf life of 8 days, which was longer than other treatments (6 days) based on microbial counts. These impacts on the bread product are due to activity of *Lc. lactis* subsp. *lactis* and its metabolites which are produced during fermentation of BM. The results of the sensory evaluation revealed that there was no change from the sensory attributes of all treatments. All crumpets accepted by panellists.

Our findings from this study confirm that BM fermented with *Lc. lactis* subsp. *lactis* which was used for crumpet breads as a model successfully improved the safety and quality, decreased Aw, delayed staling and extended shelf life of

crumpet breads by reducing the growth of total microbial counts, moulds and yeasts and growth of *Bacillus cereus*. Therefore, it might be interesting to investigate the diversity of LAB and isolate them in sourdough, which helps to understand their role in spontaneously fermented ecosystem. Further study needed such as, antimicrobial activity of isolates against food pathogenic microorganisms to achieve the effect of starter cultures on the quality, palatability and shelf life of other baked goods.

CHAPTER FIVE

Diversity of lactic acid bacteria from fermented dough – Potential use as sourdough bread starters

5.1 Introduction

Since it was found (Chapter 4) that BM fermented with LAB effectively improved the quality and shelf life of crumpet bread, when tested as a model system, it might subsequently be interesting to understand the role of LAB sourdough, which is a considerably more complex system. The diversity of LAB in such spontaneously fermented ecosystems can be explored, and that information can be used in relation to their role. An example of relevant properties of LAB is the antimicrobial activity against food pathogenic microorganisms, which can be tested in isolates. Biotechnology companies would select and evaluate potential starters to perform in fermentation to achieve optimum quality, palatability and shelf life of bread product.

Sourdough is a mixture of flour and water fermented by LAB and yeast (Vogel *et al*., 1999; Corsetti and Settanni, 2007). The levels of the LAB are usually higher than $5x10^8$ CFU/g (Ottogalli *et al*., 1996; De Vuyst and Neysens, 2005; Hansen, 2012). Sourdough is a very complex microbial ecosystem. The sourdough is used to improve nutritional value, sensory properties and to extend the shelf life of bread products because of the activity of LAB and their metabolites during the souring of the dough (Katina *et al*., 2006b; Dal Bello *et al*., 2007; Valerio *et al*., 2008; Kim *et al*., 2009). Reduction in pH is related to the production of organic acids, causing an increase in the proteases and amylases activity of the flour. This consequently reduces bread staling. While improving

the textural qualities of bread, sourdough fermentation also results in increased mineral bioavailability and reduced phytate content (Arendt *et al*., 2007).

Many species of LAB have been isolated from sourdough using culture media. Also LAB have been identified by conventional physiological and biochemical methods, and through PCR fingerprinting analysis by DNA extraction from the pellets of pure bacterial cultures (Ravyts and De Vuyst, 2011). More LAB species occur in wheat and rye flour, including strains of *Lactobacillus, Pediococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Weisella* (Hammes and Vogel, 1995; Ehrmann and Vogel, 2005; De Vuyst and Neysens, 2005; Robert *et al*., 2006). A few less than 50 different species of LAB from sourdough have been reported by De Vuyst and Neysens (2005); Hammes *et al*. (2005) and Corsetti and Settanni (2007). Additionally, the majority of LAB of the genus *Lactobacillus* have been isolated from sourdoughs (Ottogalli *et al*., 1996; Corsetti *et al*., 2001; Corsetti and Settanni, 2007) such as *Lactobacillus sanfranciscensis*, *Lb. brevis and Lb. plantarum* (Gobbetti, 1998; Corsetti *et al*., 2001, Corsetti *et al*., 2003).

Complex fermentations involve several organisms growing in succession and sharing the same environment, but it is not always possible to isolate them as they would not grow on culture conditions. Therefore, alternative non-cultural approaches can be used.

DGGE (denaturing gradient gel electrophoresis) of PCR amplicons of a fragment of the 16S rRNA gene (16S rRNAPCR-DGGE) is the basis for a molecular method which has been used to determine the variation of bacterial

populations in sourdoughs (Corsetti *et al*., 2001; De Vuyst and Vancanneyt, 2007).

The aim of this study was to assess the biodiversity of LAB strains from sourdough collection through 16S rRNA PCR-DGGE, to isolate LAB from sourdough samples using culture media and identify pure bacterial cultures by physiological and biochemical tests and through PCR fingerprinting analysis by DNA extraction. Moreover, it aims to investigate the potential influences of isolated LAB from sourdoughs on the food pathogenic microorganisms and to test LAB for proteolytic and amylolytic enzyme activities which are important in food preservation and in flavour production. The isolated LAB can be used as a starter culture for making sourdough bread which may improve the quality and shelf life of bread product.

5.2 Materials and Methods

5.2.1 Sample collection

Sourdough and bread samples (n=18) were collected from different sources as shown in Table 5.1, to investigate the diversity of LAB and also to isolate and identify LAB from sourdough collection using physiological and biochemical tests and molecular methods. Three commercial starter cultures with specific LAB were collected from Lallemand Company, France. The cultures were Florapan L62k containing a specific hetero-fermentative LAB strain (SD1), Florapan L73K containing a specific facultative (SD2) and Florapan LA4 (K), a blend of two selected LAB and one aromatic yeast (SD3). Fourteen fermented sourdough samples (SD4 to SD17, except SD9), which were spontaneously fermented from different kind of flours, were used in this experiment. San-Francisco-style sourdough bread (SD18) was collected from a supermarket (Marks and Spencer); which was made from San-Francisco starter and yeast without any additive. It was processed with sourdough samples for understanding the presence of LAB after the baking process. The samples were stored at −20°C until DNA extraction.

Table 5.1: The label of sourdough samples collection and producer

samples	Starter culture	Kind of flour	Producer
SD1	Florapan L62k	-	
SD2	Florapan L73k	-	Lallemand, France
SD3	Florapan LA4 (K)	-	
SD4		Wholemeal rye flour	Red Dog Bakery, Black Torrington, UK
SD5		Light rye flour	
SD6		White strong flour	Cheltenham, Gloucestershire, UK
SD7		Organic whole rye flour	Divine Crust, Shanklin, on the Isle of Wight, UK
SD8		Organic whole rye flour	Bread matters, Scotland, UK
SD9	*Lb. sanfrancisco*, *Torulopsis Holmii*	organic brown rice	Yemoos, Utah 84092, United States
SD10		White strong flour	Hobbs House Bakery, South Gloucestershire, UK
SD11		Organic white bread flour	Eastcourt Manor, Gillingham, Kent, UK
SD12		Rye flour	Pure Nature GmbH, Zur Rothheck 1455743 Idar-Oberstein, Germany
SD13		Organic white flour	Sturgis, South Dakota, United States
SD14		Unbleached all-purpose flour	Gold rush sourdough, Ladson, South Carolina, United States
SD15		wholegrain rye flour	Wooloowin, QLD, Australia
SD16		Hard white flour with sourdough culture	Bakery Bits Ltd, Honiton - Devon, UK
SD17		White strong flour	Column Bake-house, Plymouth - Devon, UK
SD18		San-Francisco sourdough bread	Marks and Spencer, Plymouth - Devon, UK

5.2.2 Molecular microbial method

5.2.2.1 Bacterial DNA extraction by DNeasy mericon Food Kit method for sourdough samples

DNeasy *mericon* Food Kit (Qiagen, West Sussex, UK) was used for DNA extraction from sourdough samples. 200mg homogenised sourdough samples were placed in a sterilised micro-centrifuge tube. One ml Food Lysis Buffer and 2.5µl Proteinase K solution were added to the sourdough sample, well mixed and incubated for 30min at 60°C and shaken every 5min; the samples were cooled on ice after incubation. The samples were centrifuged at 2500xg for 5min. The maximum volume of clear supernatant from lysis tube was drawn into one micro-centrifuge tube, without disturbing the inhibitor precipitate at the bottom at the tube, and mixed by pipetting up and down several times to ensure a homogeneous solution. 500µl of chloroform was pipetted into a new microcentrifuge tube. Then 700µl of the clear supernatant pool was transferred to the microcentrifuge tube containing the chloroform and mixed for 15 seconds by vortexing, then centrifugation at 14000xg for 15min. 350µl of the upper aqueous phase of the mixture was added to 350µl of Buffer PB into a fresh microcentrifuge tube and mixed thoroughly by vortexing.

The samples were pipetted into the QIAquick spin column which was placed in a 2ml collection tube. The samples were centrifuged at 17,900xg for 1min and the flow-through discarded. Then after 500µl Buffer AW2 was added to the QIAquick spin column, it was subjected to further centrifugation at 17,900xg for 1min to discard the flow-through. The collection tubes were reused for centrifugation again at 17,900xg for 1min to dry the membrane.

The QIAquick spin column was transferred to a microcentrifuge tube and 150µl Buffer EB was pipetted directly onto the QIAquick membrane. The samples were Incubated for 1min at room temperature (15–25°C), and then centrifuged at 17,900xg for 1min to elute and stored at 4°C.

5.2.2.2 Spectrophotometric assay

The DNA concentration (ng/µl) in the samples was determined by using a Nanodrop® ND-1000 spectrophotometer at a wavelength of 230nm. DNA in Buffer EB was used as a blank to re-zero the device. The results of DNA concentrations in sourdough samples were more than 20ng/µl except SD1, SD2 and SD15 where the results were (14.5, 13.1 and 16.4) ng/µl respectively. The protein purity of 260/280 and humic acid of 260/230 were higher than 1.7 except SD1, SD9 and SD15 which were less than 1.7. The average bacterial DNA of >20ng/µl are good and protein purity and humic acid purity of >1.7 are good.

5.2.2.3 PCR amplification (polymerase chain reaction)

PCR amplification of the V3 region of 16S rRNA genes was undertaken with the reverse primer P2 (534R) (5'- ATT ACC GCG GCT GG-3') and the forward primer P3 (341F+GC) with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GG GCC TAC GGG AGG CAG CAG-3'). These primers correspond to position 341-534 in the 16S rRNA of *E. coli* which produces a fragment of 193bp. Each PCR tube contained 1µl of primer P2 and P3 (50pmol/µl, Eurofins MWG Operon, Germany), 3µl DNA template, 25µl of

Ready Mix Taq DNA polymerase (Sigma Aldrich, UK) and were made up 50µl with 20µl of PCR grade water. The PCR thermal cycling was conducted under the following conditions: 94ºC for 10min, then 30 cycles starting at 94ºC for 1min, 65ºC for 2min, and 72ºC for 3min. The annealing temperature decreased by 1ºC every second cycle until 55ºC and then remained at 55ºC for the remaining cycles.

5.2.2.4 Agarose gel electrophoresis

Eight µl of the PCR products was separated by electrophoresis on a 1.5% agarose gel (Lonza, Rockland ME, USA). A mixture of 1.35g of agarose powder and 90ml of 1x TEA buffer (Tris/ EDTA/Acid) was dissolved using a microwave for 1min with periodic mixing. Eight µl of PCR product was loaded in the wells of the gel with 2µl of loading buffer. Ten µl of the 100bp DNA ladder (Fisher, UK) was used to assess the size of DNA products. The gel was run at 90volts for 45min, and the bands were visualised with UV and photographed using a Gray scale digital camera CFW-1312M (Tokyo, Japan) in the Universal Hood II, BIO-RAD Laboratories (Milan, Italy). Figure 5.1 shows the single band of DNA templates of the samples after PCR amplification.

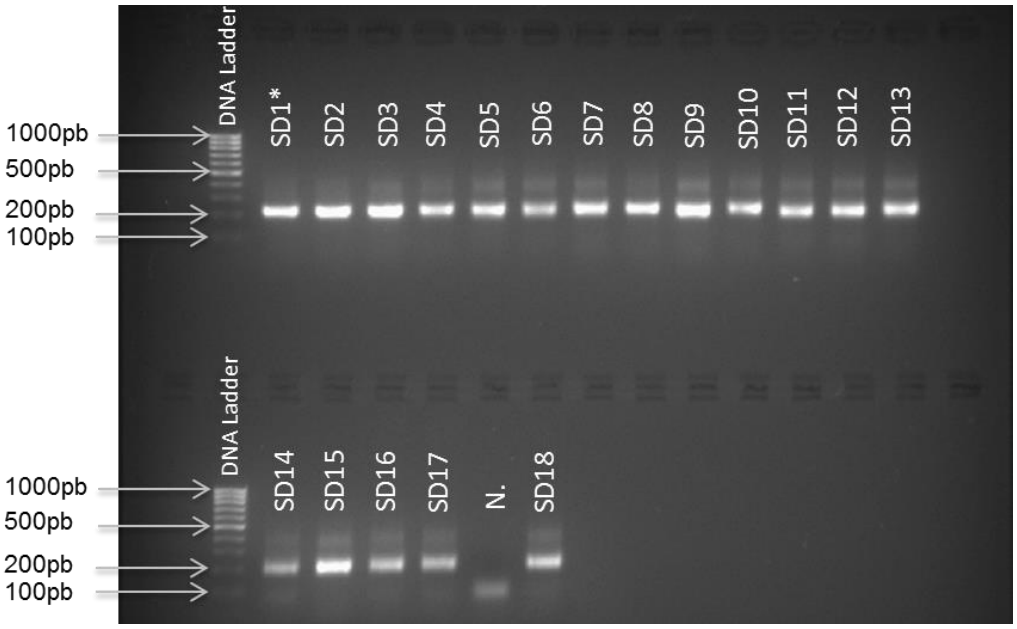


Figure 5.1: PCR amplified product of DNA templates of the samples after PCR for DGGE

* Key of the table as Table 5.1 N.: Negative

5.2.2.5 Denaturing gradient gel electrophoresis (DGGE)

The DGGE was made using a DGGE-2001 system (CBS scientific, USA). Fifteen µl of PCR products was run on acrylamide gels (16 X 16 X 0.1) cm with a denaturing gradient of 40-60% (where 100% denaturing are 7M urea and 40% formamide). A loading buffer with 200µl of gel green stain was added to the high gel solution (60%) to stain DNA in acrylamide gels. 100µl of ammonium per sulphate (APS) was added to the high and low gel solutions. 50µl of tetramethylethylindiamine (TEMED) was added to the gels and 16ml of both gel solutions were added gradually using a Bio–Rad gradient delivery system (model 475). A comb (20 wells) was inserted and gels were left for 20min to completely set. All samples were run on the same gel to prevent issues of non-

reproducibility. The outside lanes were not used. The gel was run at 60V for 16hr at 60ºC in 1x TAE buffer (66mM Tris, 5mM Na acetate, 1mM EDTA). Visualising of the DGGE band was achieved by high sensitivity and optimised gold staining method. The gel was soaked and incubated in fixation buffer (200ml 1x TAE containing 20µl SYBER safe DNA gel stain) for 25-30min on a shaking platform at room temperature, and scanned in a Bio-Rad Gel-Doc system and optimised for analysis of UV light. Bacteria were identified by sequencing PCR-DGGE fragments. DNA fragments of interest were excised aseptically from the polyacrylamide gel, under the UV light, placed in 20µl DNA grade water and incubated overnight at 4°C to allow elution of the DNA.

5.2.2.6 Preparation of samples of DGGE bands for sequencing

Five µl of diffusion DNA bands kept in molecular grade water overnight were added to a master mix which included 12.5µl of Ready Mix Taq polymerase (Sigma Aldrich, UK). 1µl of reverse primer P2 (534R) and 1µl of forward primer P1 (341F-GC), which have no GC clamp, were completed with 10.5µl of molecular grade water to make up 30µl for re-PCR products. The mixture runs using the same program of PCR-DGGE. The PCR products were cleaned after checking the concentration of PCR product by using QIAquick PCR purification kit (Qiagen, USA) to clean PCR product according to manufacturer's instructions. Briefly, 100µl of Buffer PB was added to 20µl of the PCR product and mix. QIAquick spin columns were placed in a provided 2ml collection tubes. The samples were added to the QIAquick columns and centrifuged for 30-60sec., to bind the DNA, then, flow-through was discarded and the QIAquick

column was placed back into the same tube. 750µl of buffer PE was added to the QIAquick column and centrifuged for 30-60sec. to wash the samples. The flow-through was discarded and placed the QIAquick column back into the same tube, and then the column was centrifuged for an additional 1min. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube. 30µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane and centrifuged for 1min to elute DNA; the DNA was then stored at 4°C overnight. The concentration of DNA was determined by using gel electrophoresis. Only 5µl of diluted to 20-80ng/µl of PCR product and 5µl of one of the primers (5pmol/µl) in Eppendorf tube together was sent for sequencing centre of GATC biotechnology in Germany and the sequencing results sent via their website: http://www.gatc-biotech.com/en/index.html. All the obtained DNA sequences were manually edited using Bio Edit software (Version 7.2.5) to trim the sequence ends as their sequence lengths were varying. Each sequence was compared to those in available databases by using of the BLAST (Basic Local Alignment Search Tool) from GeneBank network services at http://blast.ncbi.nlm.nih.gov/Blast.cgi for species identification. The major steps of DGGE are presented in Figure 5.2. All 16S rRNA gene sequences were aligned using ClustalW alignment by Molecular Evolutionary Genetics Analysis (MEGA) software version 6. A phylogenetic tree of the 16S rRNA gene sequences was constructed in MEGA 6 using a neighbour joining method with a P-distance as a measure of genetic distance, and bootstrap values were calculated with 1000 replicates. The transitions and transversions substitution and homogenous pattern among lineages were chosen with uniform rates among sites.

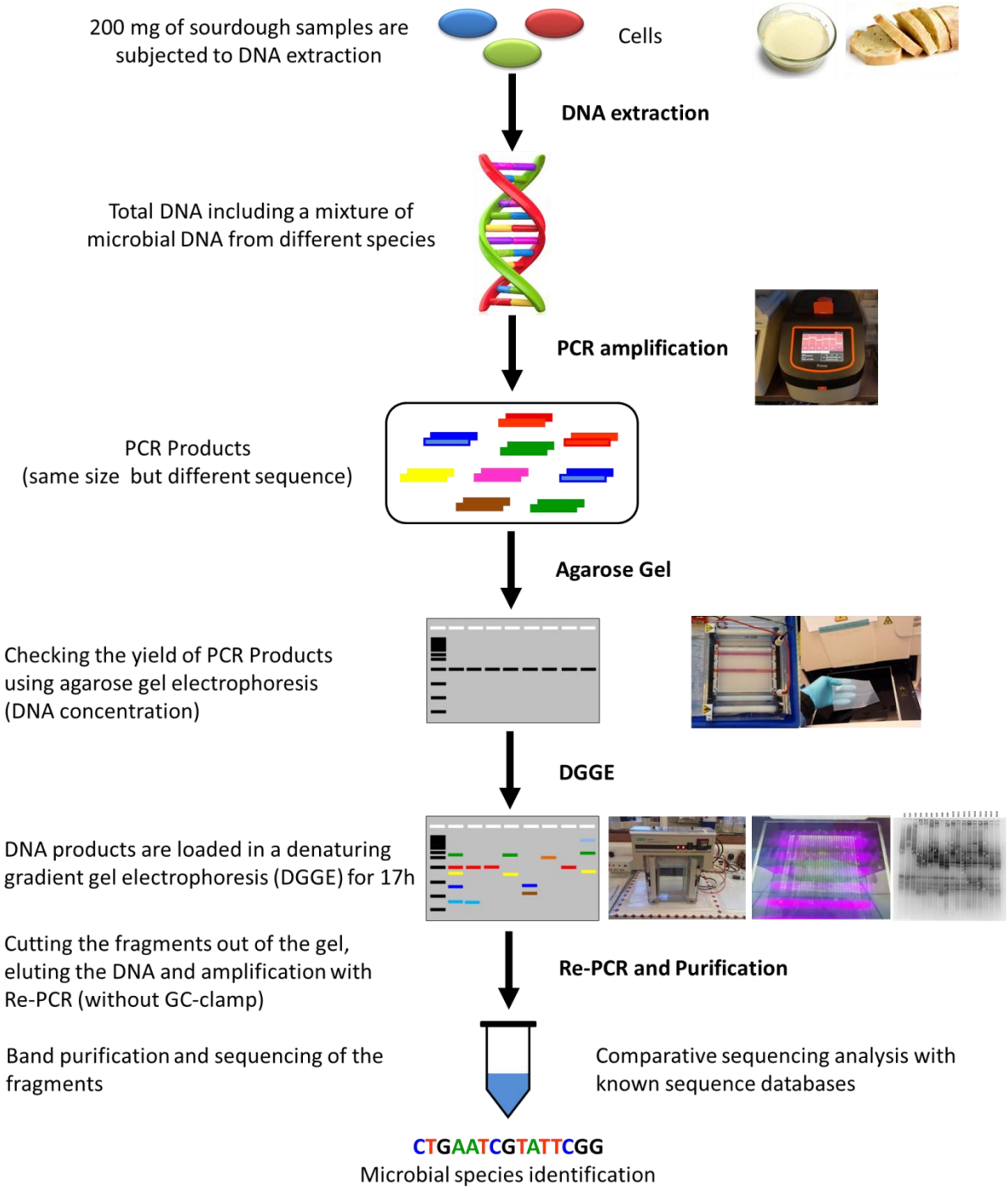


Figure 5.2: The main steps of the denaturation gradient gel electrophoresis (DGGE) process of PCR amplified DNA

5.2.3 Measurement of pH and TA values of sourdough samples

The pH and TA values of sourdough starters were determined as mentioned in Sections 2.71 and 2.7.2 respectively.

5.2.4 Preparation of culture media

5.2.4.1 Preparation of MRS media

MRS was prepared as in Section 2.3.5. This was used for growth of *Lactobacillus* species.

MRS medium was used to detect the proteolytic and amylolytic activities for LAB isolates. The MRS medium contained (per litre): 5g yeast extract (Oxoid Ltd., Basingstoke, Hampshire, England),10g peptone (Oxoid Ltd., Basingstoke, Hampshire, England),10g beef extract (Oxoid Ltd., Basingstoke, Hampshire, England), 2g K_2HPO_4 (Sigma-Aldrich, UK); 0.2g $MgSO_4$-$7H_2O$ (Sigma-Aldrich, UK); 0.05g $MnSO_4$-$4H_2O$ (Sigma-Aldrich, UK), 5g $NaHCO_3$ Sodium acetate hydrate (Sigma-Aldrich, UK), 1ml Tween 80 (Merck, Darmstadt, Germany), 12g agar, with added 2g glucose (Sigma-Aldrich, UK) for proteolytic activity and 15g starch instead glucose for amylolytic activity. The medium was adjusted to pH 6.5 then sterilised by autoclaving for 15min at 121ºC.

5.2.4.2 Preparation of Nutrient broth

Nutrient broth was prepared as in Section 2.3.1. This was used to prepare stock cultures of all pathogenic bacteria.

5.2.4.3 Preparation of skim milk (SM)

Skim milk (LP0031, Oxoid Ltd., Basingstoke, Hampshire, England) was prepared according to the manufacturers' instructions by dissolving 10% of skim milk in distilled water and autoclaved at 115°C for 10min.

5.2.4.4 Preparation of BHI agar

BHI agar was prepared as in Section 2.3.2. This was used to prepare agar plates for the agar well diffusion and agar spot methods.

5.2.5 Isolation of LAB from sourdough samples

One gram of each sourdough sample was blended with 9ml of PBS buffer solution (0.1 M, pH 7.0) and subsequently homogenised for 3min in a stomacher. The homogenate samples were serially diluted to yield dilutions of 10^{-5}, 10^{-6} and 10^{-7}, and cultured onto MRS agar medium containing 5g/L calcium carbonate. The plates were incubated in the 5% CO_2 incubator at 37°C for 48h. The resulting colonies were first characterised phenotypically by Gram-positive, catalase negative, growth at 15°C and 45°C and growth tolerance at different salt levels (2, 4 and 6.5% w/v NaCl). Gram-positive, catalase negative isolates were considered to be LAB, and then used in further studies. LAB isolates were kept in MRS broth with 30% (vol/vol) glycerol and frozen at -80°C. Stock cultures were reactivated by subculturing in MRS broth and incubating at 37°C in the 5% CO_2 incubator for 24h before use.

5.2.6 Molecular identification of LAB strains by PCR amplification and DNA sequencing of 16S rRNA

5.2.6.1 Bacterial DNA extraction

All LAB strains isolated from sourdoughs were identified by a PCR based method. DNA of each LAB isolate was extracted by using a protocol of the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Germany). The overnight cultures were centrifuged for 2min at 12000xg and the culture medium completely removed. The pellet was re-suspended thoroughly in 200µl of lysozyme solution which was prepared from lysozyme chicken egg white (L4919) diluted by 50mg/ml in TE buffer (10mM Tris-HCl, pH 8.0), and the mixture incubated for 30min at 37°C. 20µl of the proteinase K solution was added to the sample followed by 200µl of lysis solution C (B8803), vortexed thoroughly for about 15sec and then incubated at 55°C for 10min 500µl of the Column Preparation Solution was added to the pre-assembled Gen Elute Mini prep Binding Column and seated in a 2ml collection tube. The sample was centrifuged at 12000xg (Sanyo, Micro Centaur, MSE, UK) for 1min and the eluate removed. 200µl of ethanol (95-100%) was added to the sample in the lysate and mixed homogeneously for 5-10sec. The entire contents of the tube in the Load lysate was transferred into the binding column and then the sample was centrifuged at 8000xg for 1min. The collection tube containing the eluate was discarded and the column placed in a new 2ml collection tube. The first washing added 500µl wash solution 1 (W0263) to the column, and it was centrifuged for 1min at 8000xg. The collection tube containing the eluate was discarded and the column placed in a new 2ml collection tube again. The second washing added 500µl wash solution to the column, and it was

centrifuged for 3min at 12000xg to dry the column. The column was centrifuged for an additional 1min at 12000xg, because the column must be free from ethanol before eluting the DNA. Finally, the collection tube containing the eluate was discarded and the column placed in a new 2ml collection tube. The elute DNA was added in 200µl of the elution solution (B6803) directly onto the centre of the column and then incubated for 5 min at room temperature, to increase the elute efficiency. The sample was then centrifuged for 1min at 8000xg to elute the DNA. The eluate contains pure genomic DNA, and then the sample will be stored at 2-8°C for short term storage.

5.2.6.2 Spectrophotometric test

The optical density of the DNA mixture was examined using Nanodrop® ND-1000 as mentioned in Section 5.2.2.2. The DNA was measured and the average bacterial DNA were more than >20ng/µl. The protein purity of 260/280 and humic acid of 260/230 were higher than 1.7.

5.2.6.3 PCR amplification and DNA sequencing of 16S rRNA

Bacterial DNA was amplified using PCR primers obtained from (Eurofins MWG Operon, Germany);

Forward primer 27(F) 5'-AGAG TTTG ATCC TGGC TCAG-3' (20 bases)

Reverse primer 1492(R) 5'-GGCT ACCT TGTT ACGA CTT-3' (19 bases)

A mixture of 1µl of bacterial DNA extraction, with 24µl of reaction mix, containing 12.5µl Red Taq ready Mix (Sigma-Aldrich, USA), 1µl forward primers, 1µl reverse primers and 9.5µl DNA grade water was prepared in a 0.2ml PCR tube. The samples were amplified in a PCR thermal cycler (Techne, Model TC-312) for a period of 4h. The PCR program was run as follows: denature the template at 95°C for 1min, anneal primers at 55°C for 2min and extension at 72°C for 3min. Each set of reactions included a negative and a positive control.

The PCR products were then analysed by electrophoresis on a 1% agarose gel to check the size of amplicons. A mixture of 1.5g of agarose nondenaturating gel, 90ml of 1x TAE, and the agarose gel was heated briefly in the microwave with shaking for 1 min. After cooling, 4µl of SYBR®safe stain (Fisher, UK) was added to the gel. To prepare the samples for electrophoresis, eight µl of each PCR product was added to the wells. Eight µl of the 100bp DNA ladder (Fisher, UK) was used to assess the size of DNA products. The gel was run at 90volts for 45min, and the bands were visualised and photographed using a camera on a UV trans-illuminator as mentioned in Section 5.2.2.4.

5.2.6.4 DNA purification

Purification of PCR products was performed with the ChargeSwitch®-Pro PCR Clean-up Kit (invitrogen, Fisher, UK) by life technologies. All steps were performed at room temperature; three main steps were adjusted, based on the instruction of the manufacturer. Binding the DNA was achieved by adding a 1:1 volume of ChargeSwitch®-Pro PCR Purification buffer to the PCR reaction, gently vortexed to mix well. The mixture was transferred onto the

ChargeSwitch®-Pro PCR Clean-up column inserted in a collection tube. The column/tube was centrifuged at 10000xg for 1min. The column was removed from the tube and the flow-through discarded, and then the column was re-inserted in the same collection tube. The column was washed in 600µL of ChargeSwitch®-Pro PCR wash buffer. The column/tube was centrifuged at 10000xg for 1min. The flow-through and the collection tube were discarded, and the column was inserted into a new sterile elution tube. The final steps of purification were eluting the DNA. 25µL of ChargeSwitch®-Pro PCR Elution buffer was added onto the column, and incubated at room temperature for 2min. The column/tube was centrifuged at 10000xg for 1min. The flow-through contained the purified DNA. The elution step was repeated one more time, and the flow-through was collected in the same tube. The quantity of DNA purified was determined by electrophoresis assay; after dilution to 1/10 by 1µL of sample with 3µL of loading buffer and 6µL of DNA grade water, the DNA concentration was calculated by multiplying the bp of the sample with the 50bp DNA ladder (21ng/µL). Then DNA was sequenced by GATC Biotech (European Custom Sequencing Centre, Germany) and the phylogenetic tree was constructed in MEGA version 6 as mentioned in Section 5.2.2.6.

5.2.7 Measuring pH, titratable acidity (TA) values and microbial growth

One percent of each isolated LAB from an overnight culture was inoculated in MRS broth and each was put in separate flask. Then all the culture media were incubated in the incubator at 30°C for 24h. pH values were measured directly every 3h up to 24h (9 times) of the incubation period by pH meter (Corbo *et al*.,

2013). TA values were also measured every 3h of the incubation period (24h). 10ml of each sample was taken each 3h (up to 24h) of the incubation period, then titrated against 0.1N NaOH with phenolphthalein indicator. TA expressed as lactic acid percent as detailed in Section 2.7.2. Duplicate determination was made for each LAB isolate.

5.2.8 Determination of proteolytic and amylolytic enzymes activity

5.2.8.1 Determination of proteolytic activity

To detect proteolytic activity, the selected LAB strains were inoculated on plates of 80% MRS agar (2% glucose) mixed with 20% skim milk, and incubated in 5% CO_2 at 37°C for 48h followed by holding in a refrigerator (4°C) for 3 days. Protein hydrolysis was observed by the production of clear halos surrounding isolated colonies (Essid *et al*., 2009; Golshan-Tafti *et al*., 2014). This test was carried out in triplicate for each sample.

5.2.8.2 Determination of amylolytic activity

Amylolytic activity of the strains was determined by the detection of starch. MRS containing starch was used. The selected LAB strains were inoculated on MRS agar containing starch and the plates were incubated in 5% CO_2 at 37°C for 48h. After incubation, iodine reagent was added to surface of each plate to detect the presence of starch. Iodine reagent complexes with starch to form a blue-black colour in the culture medium. Clear halos surrounding colonies are indicative of

their ability to digest the starch in the medium due to the presence of alpha-amylase. This test was carried out in triplicate for each sample.

5.2.9 Detection of antagonistic activity of LAB

5.2.9.1 Preparation of broth culture bacteria

All isolated strains of LAB were cultured in MRS broth (10^7 CFU/ml) under 5% CO_2 incubator at 37ºC for 18-20h, and were used as the broth culture bacteria. In addition, food pathogenic bacteria strains such as *B. cereus* MCIMB11925*, B. subtilis, P. aeruginosa* ATCC 10817*, E. coli* ATCC 10418 and *S. aureus* ATCC 6821 were grown in nutrient broth and incubated at 37ºC for 18-20h as mentioned in Section 2.6.

5.2.9.2 Procedure for agar well diffusion method

The agar well diffusion method was used to determine the antimicrobial activity of isolated LAB from sourdough samples against bacterial strains (Kuri *et al*., 1998, Fernández-López *et al*., 2005). Stock cultures of all tested bacteria were grown in nutrient broth for 18h. Final cell concentrations were standardised to 10^7–10^8 CFU/ml using the McFarland standards as mentioned in Section 2.6. Then, 200µl of this inoculum was added to each universal tube containing 20ml molten brain heart infusion (BHI) agar, mixed well and poured into a disposable Petri dish. A sterile cork borer was used to make wells (5mm diameter) after the agar was solidified. Each well was filled with 40µl of each LAB isolates. Aliquots of fresh MRS broth were used as control. Agar plates were left for 1 hour at

room temperature to diffuse into the agar, then the plate culture were incubated at 37°C for 24h. After incubation, the diameter (mm) of the inhibition zone around the wells was measured in three directions using Vernier callipers and the average was calculated (Kuri *et al*., 1998). The assay was carried out in triplicate.

5.2.9.3 Procedure for agar spot method

To confirm the results of the agar well diffusion, an agar spot method was used. This is an *in vitro* method for evaluating antimicrobial activity such as LAB against pathogenic microorganisms. The isolated LAB strains were used for detection of antagonistic activity against the same pathogenic bacterial strains by the agar spot method according to the method of Santini *et al*., (2010). The strains of LAB were grown in MRS broth at 37°C under 5% CO_2 conditions for 24h. Three µl of each LAB overnight culture were spotted on the surface of MRS agar; plates were incubated for 24h at 37°C under 5% CO_2 conditions to allow colony spots develop. Sterile MRS broth was used as a negative control on each plate. After strain growth, an overnight broth culture of each pathogenic bacterial strain was standardised to 10^7–10^8 CFU/ml using the McFarland standards as mentioned in Section 2.6. Then a 100µl volume of this inoculum was mixed with 10ml of soft nutrient agar (0.7% w/v bacteriological agar in nutrient broth). The mixture was poured over the plate with the LAB spotted onto the surface, and left for 1h to solidify. The plates were incubated at 37°C for 24h. After incubation, the diameter (mm) of the inhibition zone around the spots was measured in three directions using Vernier callipers (the range was

±1mm) and the average was calculated (Figure 5.3). A clear zone of more than 1mm around a spot was considered as an indicator of antimicrobial effect (Tahara *et al*., 1996). Each strain was performed in triplicate.

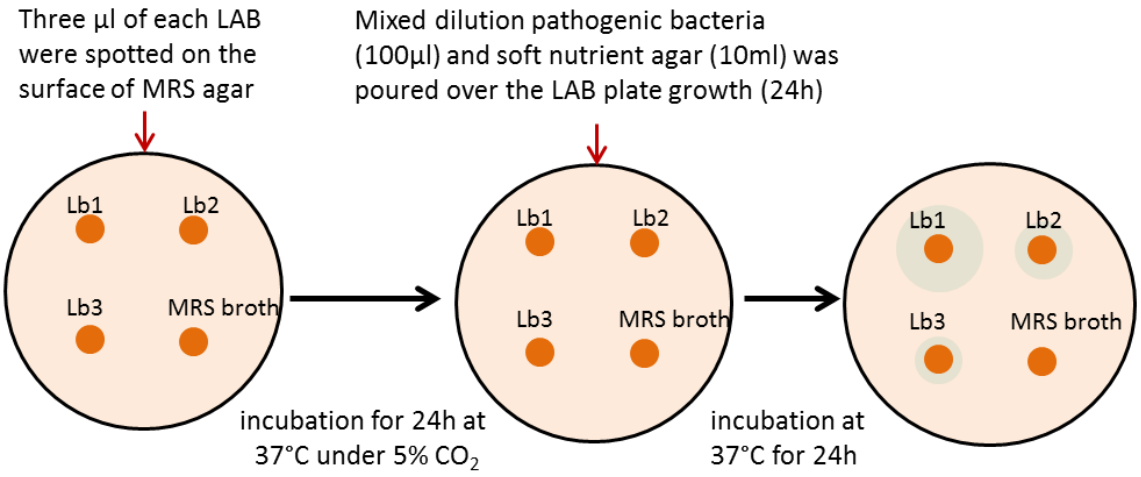


Figure 5.3: Procedure for agar spot method

5.2.10 Statistical analysis

All data were subjected to one way analysis of variance (ANOVA) as described in Section 2.9. Comparisons of sourdough microbial communities DGGE among sourdough samples were done using the Primer-6 software package (PRIMER-E Ltd, Plymouth, UK). Cluster analysis was used to check the observed groupings, and half matrix similarity analysis was also displayed as a measure of the similarity of replicates within the sourdough samples. The species richness and the microbial diversity were determined for the samples.

5.3 Results

5.3.1 PCR-DGGE DNA fingerprinting of microflora in sourdough samples

Figure 5.4 shows the PCR–DGGE bacterial profiles of the sourdough samples. Many different bands are shown in the DGGE image and the gel bands which were considered to be operative taxonomy unit (OTU) in each sample.

The similarity of bacterial populations within and between the sourdough samples was measured by using half matrix similarity (%) of sourdoughs DGGE fingerprints as shown in Table 5.2, non-metric multidimensional scaling (MDS) and cluster analysis of DGGE fingerprints as shown in Figure 5.5. There was more than 40% similarity of bacterial population between SD1, SD2 and SD3. There was about 40% similarity between all sourdough samples except SD1, SD2 and SD3. There was 60% similarity among SD4, SD5 and SD8. Also, there was about 60% similarity among SD11, SD12 SD13, SD14 SD15, SD16, SD17 and SD18 and between SD6 with SD7 as well. There was 80% similarity between each two sourdoughs (SD1 with SD3), (SD4 with SD5), (SD17 with SD18) and also there was 80% similarity among SD13, SD14 and SD16.

Figure 5.6 shows the distance of PCR-DGGE band of the sourdough samples as measured in (mm) by using image J software. The first band at the top had a distance 0.3cm from the top and the last bands in the bottom of the gel had a distance 9.43cm from the top measurement. The distance between two bands varied between 0.06-3.56cm which the smallest distance is shown between two bands in SD12 and the biggest distance is shown between two bands in SD2.

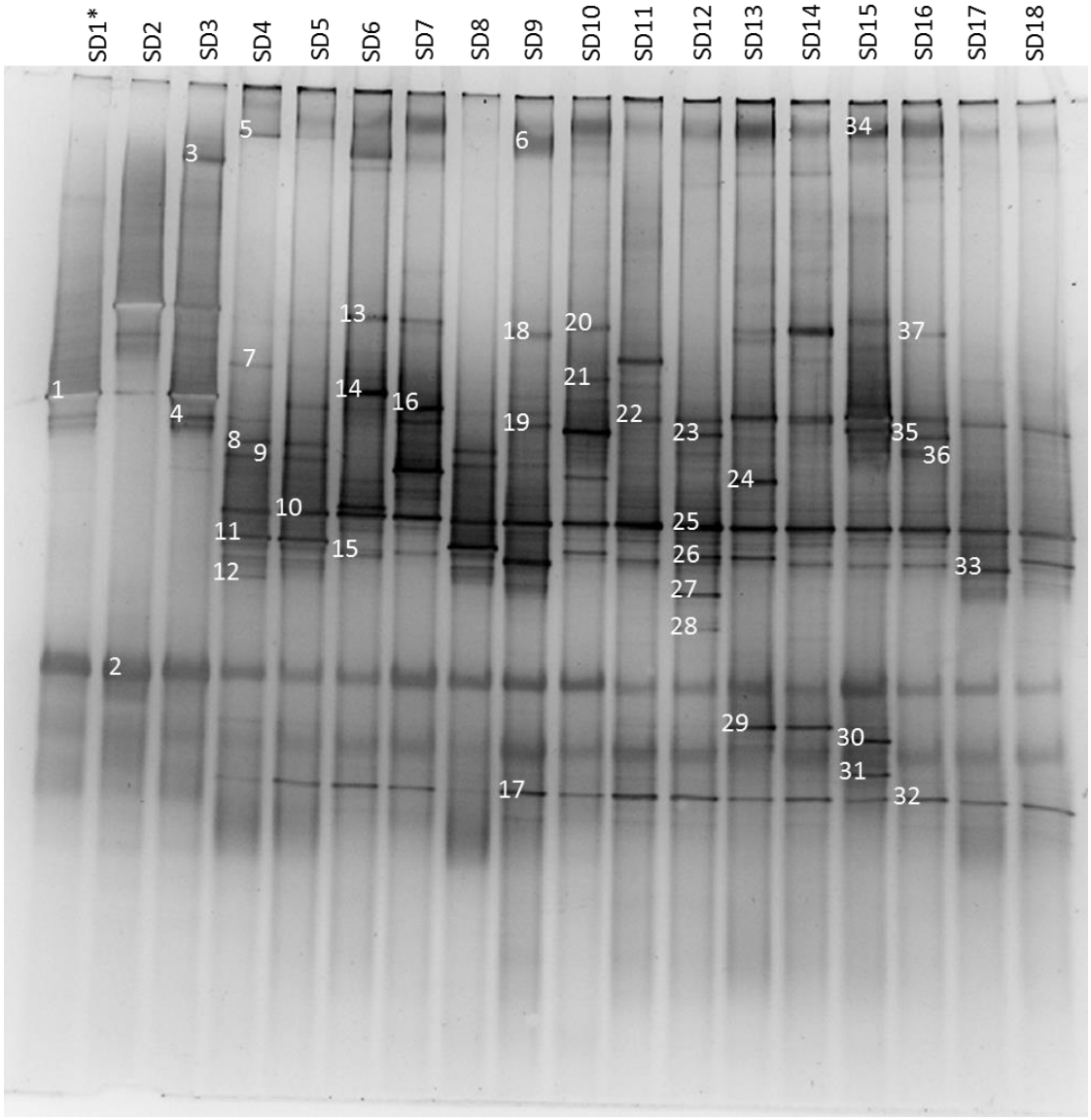


Figure 5.4: DGGE fingerprints of sourdough samples. Number of bands (operative taxonomy units (OTUs)) in each sample relates to diversity richness

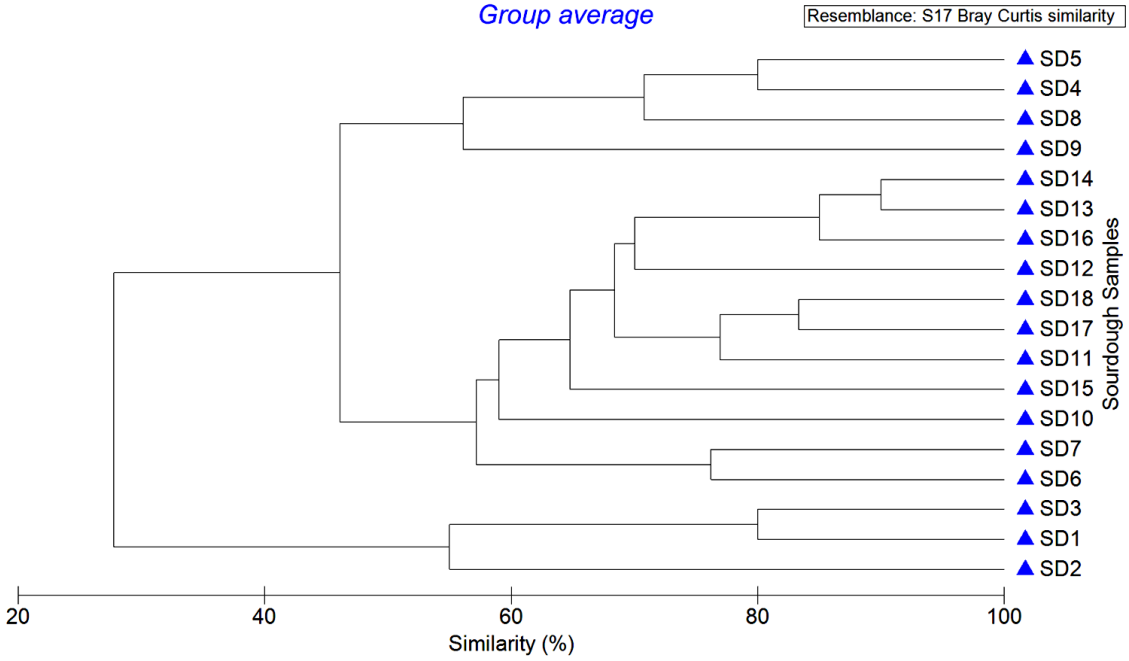
* Key of the table as Table 5.1

Table 5.2: The half matrix similarity of sourdoughs DGGE fingerprints of bacterial populations

Samples*	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	SD9	SD10	SD11	SD12	SD13	SD14	SD15	SD16	SD17	SD18
SD1	100																	
SD2	50	100																
SD3	80	60	100															
SD4	15	15	13	100														
SD5	20	20	17	80	100													
SD6	29	43	50	32	38	100												
SD7	27	27	35	30	35	76	100											
SD8	25	25	20	62	80	29	27	100										
SD9	46	15	40	56	67	42	50	46	100									
SD10	31	15	27	56	53	42	50	46	44	100								
SD11	36	18	31	38	46	59	67	36	50	50	100							
SD12	29	14	25	53	50	50	57	43	42	63	71	100						
SD13	29	14	38	53	50	50	57	43	53	63	59	60	100					
SD14	29	14	38	53	50	60	57	43	53	53	71	70	90	100				
SD15	40	27	47	40	47	57	64	40	50	60	67	67	57	67	100			
SD16	29	14	38	42	50	60	57	43	53	63	71	80	80	90	67	100		
SD17	40	20	33	40	50	63	71	40	67	53	77	62	63	63	59	63	100	
SD18	40	20	33	53	67	50	59	60	53	67	77	75	75	75	71	75	83	100

* Key of the table as Table 5.1

A



B

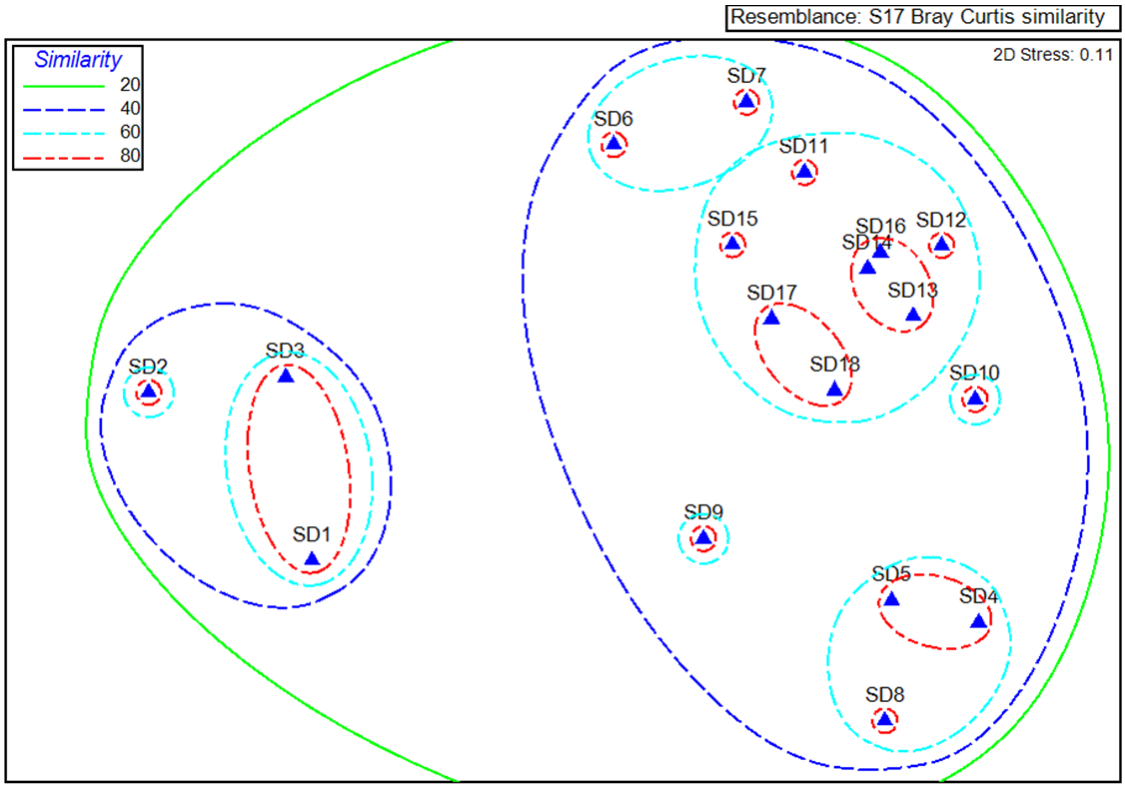


Figure 5.5: (A) Cluster analysis and (B) non-metric multidimensional scaling (MDS) graph based on the PCR-DGGE DNA fingerprints showing similarity (%) of bacterial communities between sourdough samples. * Key of the table as Table 5.1

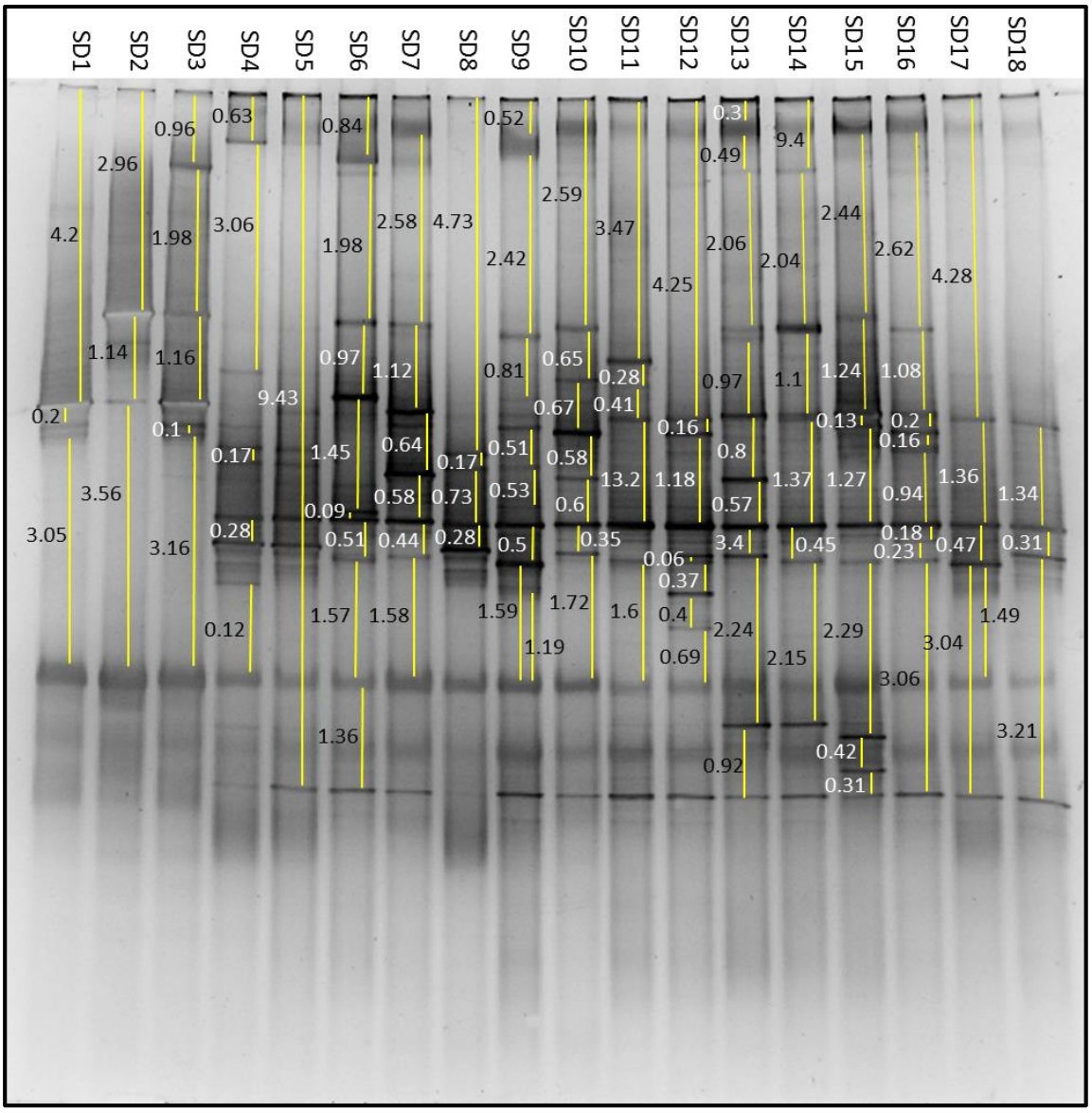


Figure 5.6: Distances of PCR-DGGE fingerprints of sourdough samples

* Key of the table as Table 5.1

Diversity and richness of sourdough samples are shown in Table 5.3. These indexes were used to display the microbial population diversity and richness in the sourdough samples. The diversity and richness of bacterial community based on the PCR-DGGE DNA fingerprinting of sourdough samples indicated that; there were differences in diversity and richness among the sourdough samples. The diversity varied between 2.16 and 4.17 and richness varied between 1.39 and 2.4. The diversity and richness of SD7 and SD15 were higher

value than the other sourdough samples which reached 4.17 and 2.4 respectively.

Table 5.3: Diversity index and richness of bacterial community in sourdough samples based on the PCR-DGGE DNA fingerprinting

Samples[1]	Band No.	Diversity[2]	Richness[3]
SD1	4	2.16	1.39
SD2	4	2.16	1.39
SD3	6	2.79	1.79
SD4	9	3.64	2.2
SD5	6	2.79	1.79
SD6	10	3.91	2.3
SD7	11	4.17	2.4
SD8	4	2.16	1.39
SD9	9	3.64	2.2
SD10	9	3.64	2.2
SD11	7	3.08	1.95
SD12	10	3.91	2.3
SD13	10	3.91	2.3
SD14	10	3.91	2.3
SD15	11	4.17	2.4
SD16	10	3.91	2.3
SD17	6	2.79	1.79
SD18	6	2.79	1.79

[1] Key of the table as Table 5.1
[2] Diversity: H" = -SUM (pi*Log (pi)).
[3] Richness: d = (S -1)/log (N).

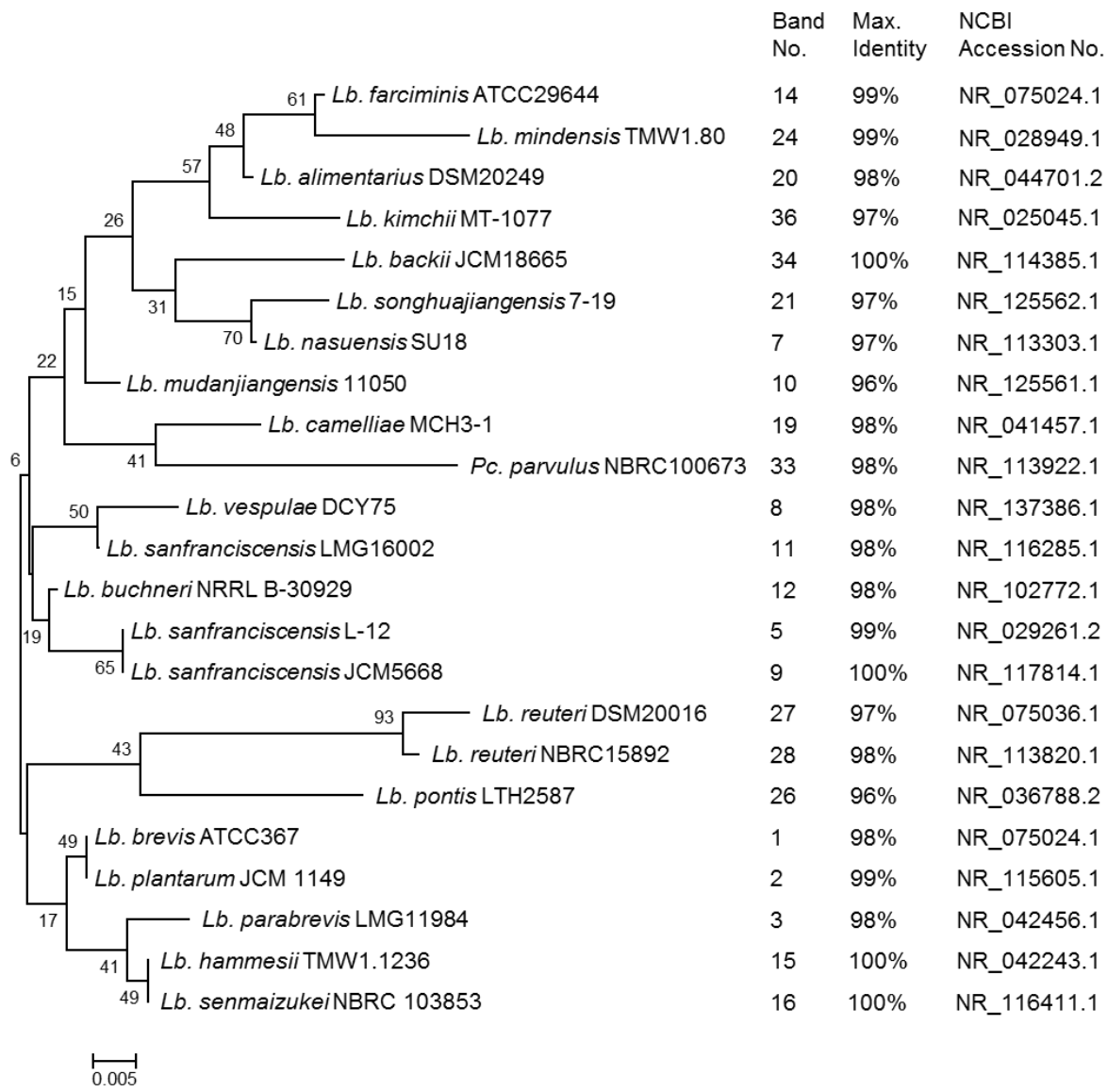


Figure 5.7: Phylogenetic tree showing the relative position of sourdough LAB based on 16S rDNA gene sequences, using the neighbour joining method. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree, using MEGA 6 software. The scale bar corresponds to 0.05 units of the number of base substitutions per site.

Figure 5.7 shows the phylogenetic relationship among LAB strains that were constructed based on the 16S rDNA sequences using the neighbour joining method. The identification of bands in PCR-DGGE fingerprints of the bacterial population of sourdough samples were selected from the bands in Figure 5.4 for

sequencing. Most of the bands that were found are *Lactobacillus* species. The maximum identity of bacterial species was varied between 96 to 100%. There were some uncultured bacteria found in the selected bands, which might be mainly as-yet-uncultured bacteria in the data banks and they were discarded due to their maximum identity which were less than 90%.

5.3.2 pH and TA values measurement of sourdough samples

The pH and TA values of the sourdough samples are shown in Table 5.4 and Figure 5.8. Three different groups were defined according to the pH and TA ranges. There were significant differences ($P<0.05$) in pH and TA values among the sourdough samples. The pH values ranged from 3.46 to 4.36 and TA values from 0.67 to 1.05mg/100mg. Thirteen sourdoughs had pH values of less than 4.0. The sourdough samples of the high acids group including SD6 and SD8 have a lower pH and higher TA values in comparison with the other sourdough samples, whereas SD9 has a higher pH and lower TA values in comparison with the other sourdough samples.

Table 5.4: pH and TA (mean*±standard deviations) of sourdough collection samples grouped by acidity

Groups	samples**	pH	TA (mg/100mg)
High acid	SD8	3.46±0.02 ^{a}	1.05±0.01 ^{a}
	SD6	3.48±0.02 ^{a}	1.05±0.01 ^{a}
Medium acid	SD4	3.53±0.03 ^{b}	0.88±0.02 ^{d}
	SD13	3.57±0.03 ^{bc}	0.92±0.02 ^{b}
	SD10	3.61±0.02 ^{c}	0.88±0.02 ^{d}
	SD2	3.69±0.02 ^{d}	0.78±0.01 ^{fg}
	SD11	3.75±0.01 ^{e}	0.82±0.02 ^{e}
	SD3	3.75±0.01 ^{e}	0.89±0.02 ^{cd}
	SD7	3.81±0.03 ^{f}	0.91±0.01 ^{bc}
	SD16	3.88±0.01 ^{g}	0.83±0.01 ^{e}
	SD1	3.92±0.02 ^{gh}	0.71±0.01 ^{hi}
	SD15	3.94±0.02 ^{h}	0.80±0.01 ^{ef}
	SD12	3.96±0.02 ^{h}	0.94±0.02 ^{b}
	SD14	4.06±0.02 ^{i}	0.75±0.02 ^{gh}
Low acid	SD5	4.23±0.02 ^{j}	0.69±0.01 ^{ij}
	SD17	4.29±0.02 ^{k}	0.71±0.02 ^{hi}
	SD9	4.36±0.02 ^{l}	0.67±0.01 ^{j}

* $^{a\text{-}l, a\text{-}j}$ Means (n=3) with different letters are significantly different (P<0.05).

** Key of the table as Table 5.1

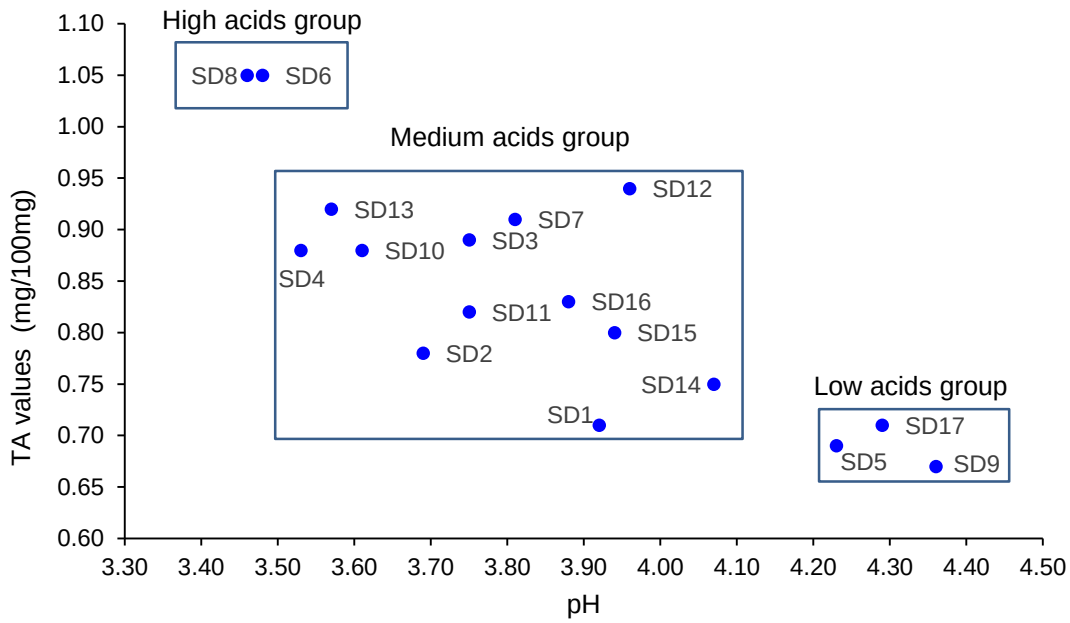


Figure 5.8: The pH and TA values of sourdough samples

5.3.3 LAB isolates from sourdough samples

Thirty two isolates of LAB were isolated from seventeen sourdough samples as shown in Table 5.5. All LAB isolates of clear zones on MRS agar plates were randomly selected and purified. Phenotypic and biochemical characterisation was carried out for identification of LAB. All isolates were Gram positive, catalase negative. All LAB isolates grew at 15ºC and salt levels 2 and 4% w/v NaCl. 26 isolates grew at 45ºC, 28 isolates grew in salt level 6.5% w/v NaCl and Lb20 were not grown at both characters as the results are shown in Table 5.6.

Table 5.5: LAB isolates with their sources

Number of isolates	Isolates	Source
1	Lb1	SD1
1	Lb2	SD2
2	Lb3 and Lb4	SD3
3	Lb5, Lb6 and Lb7	SD4
3	Lb8, Lb9 and Lb10	SD5
1	Lb11	SD6
2	Lb12 and Lb13	SD7
1	Lb14	SD8
2	Lb15 and Lb16	SD9
2	Lb17 and Lb18	SD10
3	Lb19, Lb20 and Lb21	SD11
1	Lb22	SD12
3	Lb23 Lb24 and Lb25	SD13
1	Lb26	SD14
2	Lb27 and Lb28	SD15
1	Lb29	SD16
3	Lb30, Lb31and Lb32	SD17

Table 5.6: Characterisation of LAB isolates from sourdough samples

Number of isolates	Isolates	Growth at 45ºc	Growth at NaCl 6.5%
23	Lb1, Lb2, Lb3, Lb4, Lb6, Lb8, Lb9, Lb11, Lb12, Lb14, Lb15, Lb17, Lb18, Lb19, Lb21, Lb22, Lb23, Lb25, Lb26, Lb27, Lb29, Lb30 and Lb32	+	+
5	Lb5, Lb7, Lb10, Lb16 and Lb31	-	+
3	Lb13, Lb24 and Lb28	+	-
1	Lb20	-	-

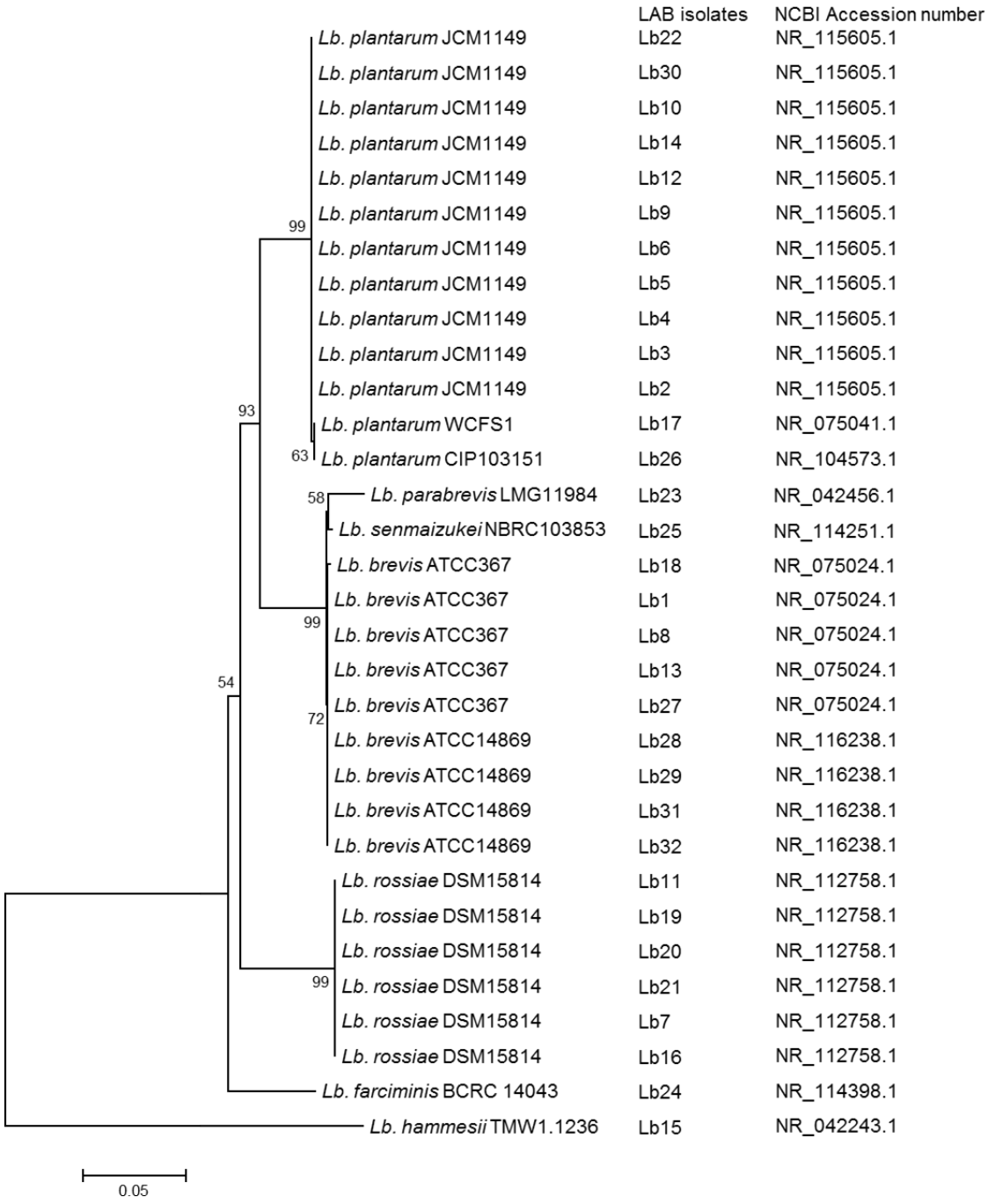


Figure 5.9: Phylogenetic tree showing the relative position of LAB isolates from sourdoughs based on 16S rDNA gene sequences, using the neighbour joining method. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree, using MEGA 6 software. The scale bar corresponds to 0.05 units of the number of base substitutions per site.

5.3.4 Identification of isolates LAB from sourdough samples

Figure 5.9 shows the phylogenetic relationship among LAB strains, constructed based on the 16S rDNA sequences using neighbour joining method. All isolated LAB from sourdoughs belong to the family *Lactobacillaceae*, where thirteen of them are *Lactobacillus plantarum*, nine are *Lactobacillus brevis* and six are *Lactobacillus rossiae* DSM15814. Lb15 are *Lactobacillus hammesii* strain TMW 1.1236. Lb23 are *Lactobacillus parabrevis* strain LMG 11984, Lb24 are *Lactobacillus farciminis* strain BCRC 14043. Lb25 are *Lactobacillus senmaizukei* strain NBRC 103853. The maximum identity of bacterial species was 99%.

5.3.5 pH and acidity development of LAB in MRS broth

The pH and TA values during the incubation period of the media culture fermented with LAB isolates are shown in Table 5.7 and Table 5.8 respectively. The pH levels varied between 3.63 and 4.30 after 24 h. Eighteen LAB strains had pH values of less than 4.0. The TA values of the LAB strains were between 0.57 and 1.13 mg/100mg after 24h of the incubation period.

Three different groups were detected according to the pH and TA as shown in the Figure 5.10. LAB strains of the high acids group include Lb1, Lb2, Lb3, Lb11, Lb14, Lb17 which had lower pH values and higher TA values. pH and TA values of LAB strains of the medium acids group varied between 3.78 and 3.92 and 0.78 and 0.97mg/100mg respectively after 24h of incubation. LAB strains of the low acids group had pH values above 4.0 and TA values varied between 0.57 and 0.74mg/100mg.

Table 5.7: pH development by the LAB isolates in MRS broth media over 24h incubation at 30ºC

LAB isolates	Incubation time (h)								
	0	3	6	9	12	15	18	21	24
Lb14	6.00	5.68	5.10	4.42	4.18	4.01	3.81	3.73	3.63
Lb11	6.01	5.60	5.05	4.54	4.15	4.00	3.87	3.73	3.67
Lb17	5.97	5.81	5.50	4.83	4.10	3.97	3.89	3.81	3.72
Lb2	6.00	5.49	5.08	4.76	4.21	4.09	3.90	3.80	3.73
Lb1	6.00	5.60	5.28	4.79	4.31	4.10	3.95	3.84	3.76
Lb3	6.05	5.75	5.33	4.89	4.30	4.14	4.01	3.89	3.77
Lb7	6.02	5.72	5.46	5.09	4.91	4.45	4.09	3.90	3.78
Lb23	6.05	5.93	5.65	4.88	4.27	4.02	3.93	3.87	3.80
Lb22	6.01	5.87	5.46	4.99	4.35	4.05	3.96	3.89	3.81
Lb5	5.98	5.45	5.10	4.93	4.45	4.24	4.05	3.91	3.81
Lb25	5.99	5.86	5.36	5.06	4.82	4.31	4.07	3.95	3.82
Lb28	6.02	5.75	5.53	5.31	4.81	4.38	4.08	3.93	3.82
Lb12	6.02	5.72	5.45	5.21	5.00	4.71	4.29	4.05	3.84
Lb30	6.00	5.84	5.58	5.21	4.79	4.14	3.96	3.89	3.84
Lb26	5.99	5.81	5.42	4.95	4.55	4.14	3.98	3.91	3.85
Lb9	6.04	5.69	5.33	5.00	4.51	4.17	4.02	3.90	3.86
Lb6	6.03	5.69	5.40	5.02	4.81	4.33	4.12	4.00	3.90
Lb4	6.00	5.67	5.32	4.98	4.61	4.43	4.23	4.05	3.92
Lb16	6.03	5.76	5.58	5.30	5.07	4.79	4.50	4.22	4.01
Lb24	6.01	5.87	5.56	5.12	4.95	4.72	4.32	4.18	4.02
Lb10	6.03	5.82	5.64	5.43	5.07	4.81	4.57	4.29	4.09
Lb15	6.02	5.89	5.76	5.49	5.29	4.85	4.49	4.26	4.11
Lb32	6.03	5.91	5.78	5.51	5.02	4.71	4.54	4.27	4.14
Lb18	5.99	5.93	5.81	5.51	4.89	4.53	4.35	4.25	4.18
Lb8	5.99	5.81	5.63	5.47	5.24	5.02	4.73	4.49	4.20
Lb19	6.04	5.95	5.86	5.44	4.85	4.51	4.37	4.24	4.20
Lb31	6.04	5.93	5.79	5.54	5.11	4.83	4.68	4.39	4.21
Lb21	6.04	5.96	5.80	5.52	4.83	4.55	4.42	4.37	4.24
Lb27	6.01	5.84	5.75	5.46	5.02	4.79	4.54	4.37	4.25
Lb29	6.01	5.90	5.76	5.46	5.10	4.87	4.64	4.42	4.28
Lb13	6.02	5.84	5.68	5.53	5.41	5.13	4.89	4.58	4.29
Lb20	6.03	5.94	5.85	5.54	4.83	4.57	4.41	4.37	4.30
P. value	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.02

Table 5.8: TA production (mg/100mg) by the LAB isolates in MRS broth media over 24h incubation at 30ºC

Acid group	LAB isolates	Incubation time (h)								
		0	3	6	9	12	15	18	21	24
High acid	Lb14	0.21	0.32	0.44	0.50	0.59	0.67	0.79	0.96	1.13
	Lb2	0.22	0.29	0.36	0.48	0.55	0.62	0.74	0.86	1.08
	Lb1	0.21	0.27	0.32	0.38	0.44	0.55	0.67	0.84	1.05
	Lb3	0.21	0.28	0.35	0.45	0.53	0.60	0.69	0.82	1.04
	Lb11	0.21	0.31	0.41	0.47	0.52	0.62	0.70	0.85	1.03
	Lb17	0.19	0.31	0.35	0.41	0.53	0.62	0.66	0.82	1.01
Medium acid	Lb4	0.21	0.27	0.30	0.36	0.41	0.50	0.58	0.74	0.97
	Lb12	0.20	0.30	0.36	0.48	0.54	0.61	0.72	0.82	0.94
	Lb23	0.21	0.27	0.32	0.37	0.54	0.58	0.65	0.79	0.94
	Lb7	0.19	0.26	0.31	0.36	0.46	0.56	0.66	0.77	0.92
	Lb22	0.21	0.25	0.28	0.34	0.49	0.56	0.63	0.72	0.89
	Lb30	0.18	0.26	0.33	0.39	0.54	0.62	0.69	0.77	0.89
	Lb5	0.22	0.25	0.31	0.36	0.41	0.47	0.59	0.72	0.88
	Lb6	0.20	0.27	0.32	0.39	0.45	0.53	0.58	0.67	0.86
	Lb25	0.19	0.28	0.30	0.36	0.51	0.60	0.67	0.73	0.85
	Lb9	0.20	0.27	0.35	0.43	0.49	0.56	0.64	0.73	0.84
	Lb28	0.19	0.29	0.32	0.41	0.51	0.56	0.70	0.74	0.83
	Lb26	0.18	0.26	0.28	0.33	0.56	0.63	0.69	0.72	0.78
Low acid	Lb10	0.19	0.24	0.31	0.38	0.45	0.52	0.60	0.67	0.74
	Lb16	0.20	0.27	0.33	0.39	0.45	0.51	0.58	0.65	0.73
	Lb24	0.18	0.30	0.32	0.40	0.49	0.56	0.62	0.66	0.72
	Lb8	0.20	0.28	0.31	0.36	0.44	0.53	0.60	0.66	0.71
	Lb19	0.21	0.24	0.29	0.37	0.44	0.50	0.57	0.64	0.71
	Lb27	0.21	0.26	0.36	0.38	0.43	0.46	0.56	0.62	0.71
	Lb13	0.19	0.24	0.32	0.36	0.43	0.48	0.55	0.63	0.69
	Lb31	0.21	0.28	0.31	0.36	0.41	0.48	0.54	0.59	0.67
	Lb32	0.19	0.29	0.32	0.44	0.51	0.54	0.58	0.61	0.67
	Lb15	0.19	0.22	0.28	0.34	0.41	0.48	0.54	0.58	0.66
	Lb18	0.18	0.26	0.37	0.40	0.47	0.51	0.54	0.60	0.65
	Lb20	0.18	0.31	0.35	0.40	0.43	0.47	0.51	0.54	0.59
	Lb29	0.20	0.24	0.29	0.36	0.43	0.45	0.48	0.51	0.59
	Lb21	0.20	0.27	0.30	0.35	0.37	0.42	0.48	0.53	0.57
P. value		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

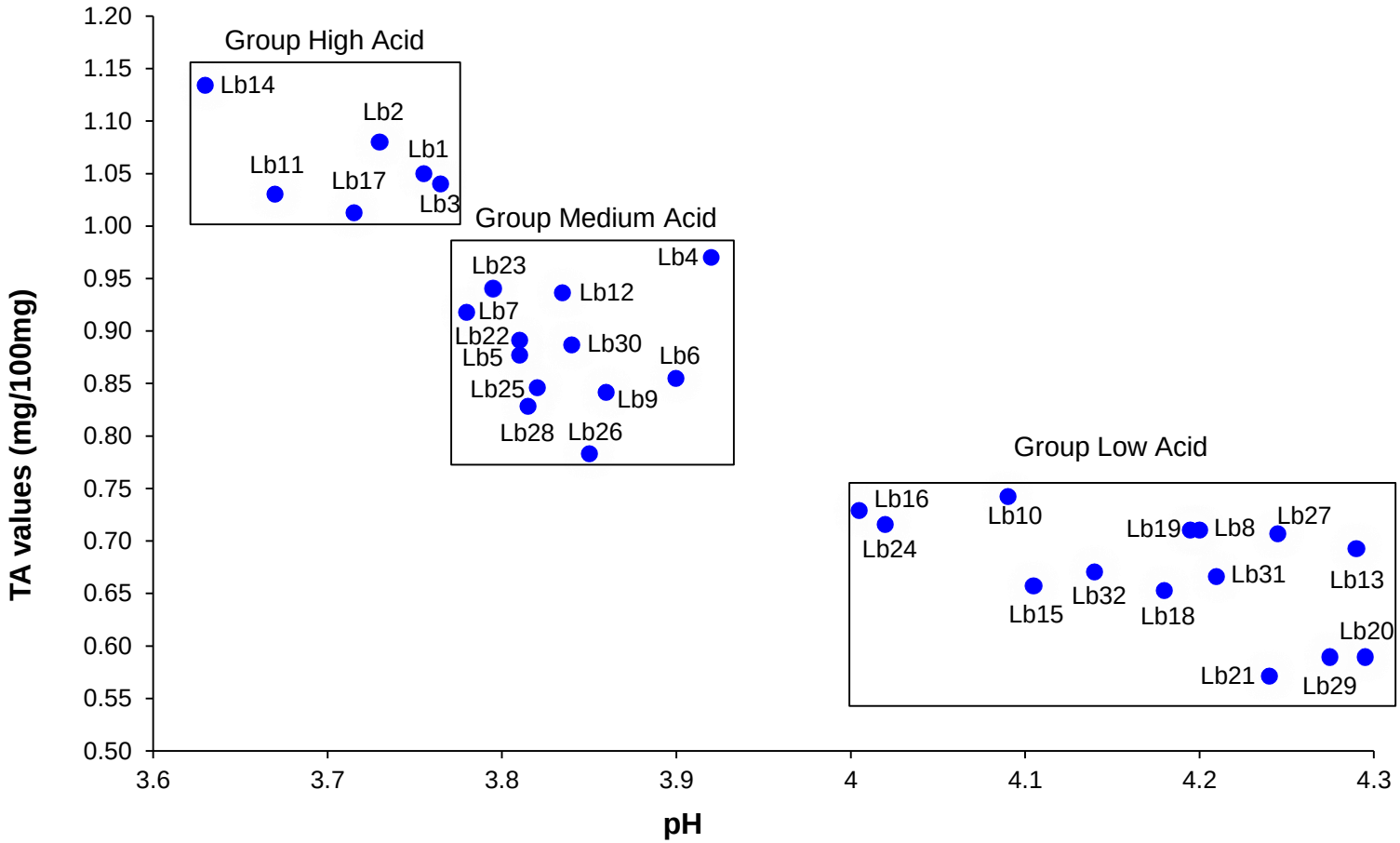


Figure 5.10: pH and TA values of isolated LAB from sourdough samples discriminating the cultures by acidity production level at 30°C for 24h

5.3.6 Proteolytic and amylolytic enzyme activities of LAB strains

Twelve isolates of the LAB presented proteolytic activity and ten LAB strains presented amylolytic activity as shown in Table 5.9. From six LAB stains of high acid group, five of them had proteolytic activity. Four LAB strains from six strains of a high acid group had amylolytic activity, while three of the LAB strains from twelve of a medium acid group had proteolytic and amylolytic enzyme activities. Four and three of LAB strains from fourteen LAB strains of a low acid group had proteolytic and amylolytic enzyme activities respectively.

Table 5.9: Proteolytic and amylolytic enzyme activities of LAB strains

Acidity groups	Enzyme activities	
	Proteolytic activity	Amylolytic activity
High acid	Lb1, Lb2, Lb3, Lb11, Lb14	Lb1, Lb2, Lb11, Lb14
Medium acid	Lb5, Lb12, Lb23	Lb28, Lb4, Lb30
Low acid	Lb8, Lb19, Lb20, Lb27	Lb15, Lb20, Lb27

5.3.7 Microbial growth

The cell densities (OD_{595}) of LAB in MRS broth media over 24h of incubation period are shown in the Table 5.10. The range of bacterial growth over 24h incubation period varied between 1.22 and 1.88. All LAB strains of the high acids group had a good growth at 24h of incubation at 30°C which varied between 1.78 and 1.82. The range of LAB growth of the medium acid group varied between 1.56 and 1.88, while the low acid group varied between 1.22 and 1.77.

Table 5.10: Bacterial growth (OD_{595}) over 24h of incubation period at 30°C in MRS broth media

Acid group	LAB isolates	Incubation time (h)				
		0	6	12	18	24
High acid	Lb14	0.22	0.53	1.07	1.62	1.82
	Lb17	0.20	0.37	1.19	1.65	1.82
	Lb1	0.23	0.52	1.19	1.67	1.81
	Lb11	0.26	0.50	1.15	1.67	1.81
	Lb2	0.21	0.48	1.14	1.66	1.79
	Lb3	0.20	0.40	1.03	1.65	1.78
Medium acid	Lb30	0.22	0.54	1.23	1.60	1.88
	Lb26	0.21	0.54	1.37	1.76	1.87
	Lb28	0.19	0.37	1.16	1.52	1.82
	Lb6	0.22	0.47	1.19	1.66	1.80
	Lb5	0.23	0.45	1.13	1.66	1.79
	Lb9	0.31	0.49	1.11	1.68	1.79
	Lb25	0.19	0.51	1.46	1.64	1.78
	Lb22	0.21	0.34	0.93	1.52	1.76
	Lb23	0.19	0.53	1.29	1.65	1.76
	Lb7	0.21	0.51	1.02	1.58	1.75
	Lb4	0.21	0.51	1.05	1.53	1.72
	Lb12	0.22	0.34	0.83	1.29	1.56
Low acid	Lb10	0.22	0.30	0.97	1.66	1.77
	Lb19	0.19	0.30	0.85	1.21	1.69
	Lb32	0.20	0.37	1.02	1.44	1.67
	Lb16	0.22	0.42	0.71	1.18	1.66
	Lb24	0.20	0.43	1.25	1.58	1.64
	Lb27	0.19	0.30	1.02	1.41	1.62
	Lb29	0.20	0.25	0.94	1.35	1.58
	Lb31	0.19	0.35	0.97	1.36	1.56
	Lb20	0.18	0.31	0.77	1.34	1.53
	Lb21	0.18	0.29	0.78	1.18	1.51
	Lb18	0.18	0.27	0.41	0.82	1.41
	Lb15	0.19	0.24	0.42	1.16	1.39
	Lb8	0.22	0.31	0.44	0.88	1.28
	Lb13	0.16	0.20	0.31	0.61	1.22
P. value		0.01	0.01	0.02	0.01	0.01

5.3.8 Antagonistic activity of LAB of sourdough samples

The antibacterial activity of LAB isolates were tested against bacterial strains such as *B. cereus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* by agar well diffusion and agar spot methods; the results are shown in Table 5.13 and 5.14 respectively. The LAB strains gave zones of inhibition against the bacterial strains. Most of LAB strains were able to inhibit the growth of bacterial strains to varying degrees.

The results of agar well diffusion method are in Table 5.11. All isolates of LAB exhibited different inhibitory activity. Lb1, Lb2, Lb11, Lb12 and Lb14 strains had the strongest (14-17mm) diameter zones against all bacterial strains except Lb2, which had intermediate (10–13mm) diameter zones against *S. aureus*, and Lb12, which had intermediate diameter zones against *P. aeruginosa* and *S. aureus*. However, other LAB isolates strains had intermediate, weak (6-9mm) diameter zones or no inhibitory activities against bacterial strains (Table 5.13).

To further confirm the results of agar well diffusion method, the agar spot method was used and the results are shown in Table 5.12. Lb1 and Lb2 had the strongest (14-17mm) diameter zones against *E. coli*, *P. aeruginosa* and *S. aureus,* and intermediate (10–13mm) diameter zones against *B. cereus* and *B. subtilis*. Lb11 had the strongest diameter zones against all bacterial strains except *E. coli*. Lb14 had the strongest diameter zones against *B. cereus*, *P. aeruginosa* and *S. aureus*, and intermediate diameter zones against *B. subtilis* and *E. coli*. Other isolates had intermediate or weak diameter zones or no inhibitory activities against bacterial strains.

As the results of antibacterial activities show, Lb1, Lb2, Lb11, Lb12 and Lb14 strains had higher antibacterial activities than the other strains of LAB which isolated from sourdough samples.

Table 5.11: Antibacterial activity* of LAB strains against some bacterial strains using an agar well diffusion method

Isolates	Target strains					Ranking"
	B. cereus	*B. subtilis*	*E. coli*	*P.aeruginosa*	*S. aureus*	
Lb1	+++	+++	+++	+++	+++	15
Lb11	+++	+++	+++	+++	+++	15
Lb14	+++	+++	+++	+++	+++	15
Lb2	+++	+++	+++	+++	++	14
Lb12	++	+++	+++	++	++	12
Lb4	++	+++	++	++	++	11
Lb23	++	+++	++	++	++	11
Lb3	++	++	++	++	++	10
Lb5	++	++	++	++	++	10
Lb24	++	++	++	++	++	10
Lb19	+	++	++	++	++	9
Lb6	++	++	+	+	++	8
Lb28	+	++	+	++	++	8
Lb10	++	++	++	-	+	7
Lb20	+	++	++	+	+	7
Lb21	+	++	+	+	++	7
Lb25	++	++	+	+	+	7
Lb30	+	++	++	+	+	7
Lb32	+	+	+	++	++	7
Lb15	++	++	++	-	-	6
Lb22	+	++	++	+	-	6
Lb9	+	++	+	-	+	5
Lb17	-	+	+	-	++	4
Lb27	+	++	+	-	-	4
Lb31	+	++	-	-	+	4
Lb7	-	-	+	+	+	3
Lb8	-	-	+	+	+	3
Lb13	-	-	+	+	+	3
Lb16	+	+	-	+	-	3
Lb18	-	-	+	+	+	3
Lb26	+	-	-	+	+	3
Lb29	-	+	-	+	+	3

* Diameter of inhibition zone: (–) no inhibition zone, (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm) and (+++) strong (14 – 16 mm). Mean values from three replicates. " Degree of ranking from more to less

Table 5.12: Antibacterial activity* of LAB strains against some bacterial strains using the agar spot method

Isolates	Target strains					Ranking"
	B. cereus	*B. subtilis*	*E. coli*	*P.aeruginosa*	*S. aureus*	
Lb11	+++	+++	++	+++	+++	14
Lb1	++	++	+++	+++	+++	13
Lb14	+++	++	++	+++	+++	13
Lb2	++	++	++	+++	++	11
Lb3	++	++	++	++	++	10
Lb23	+	++	+	+	++	7
Lb4	+	+	++	+	+	6
Lb5	+	+	+	+	++	6
Lb12	+	++	+	+	+	6
Lb6	+	+	+	+	+	5
Lb19	+	+	+	+	+	5
Lb21	+	+	+	+	+	5
Lb24	+	+	+	+	+	5
Lb25	+	+	+	+	+	5
Lb28	+	+	+	+	+	5
Lb30	+	+	+	+	+	5
Lb20	+	+	+	-	+	4
Lb32	-	+	+	+	+	4
Lb7	-	-	+	+	+	3
Lb9	+	+	+	-	-	3
Lb10	+	+	+	-	-	3
Lb15	+	+	+	-	-	3
Lb17	+	+	+	-	-	3
Lb18	-	-	+	+	+	3
Lb22	+	+	+	-	-	3
Lb27	+	+	+	-	-	3
Lb31	+	+	-	-	+	3
Lb8	-	-	-	+	+	2
Lb13	-	-	+	-	+	2
Lb26	-	-	-	+	+	2
Lb29	-	+	-	-	+	2
Lb16	-	-	-	-	-	0

* Diameter of inhibition zone: (–) no inhibition zone, (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm) and (+++) strong (14 – 16 mm). Mean values from three replicates. Degree of ranking from more to less

As the result of antimicrobial activity, enzyme activities and acidification, Lb1 (*Lactobacillus brevis* ATCC 367), Lb11 (*Lactobacillus rossiae* strain DSM 15814) and Lb14 (*Lactobacillus plantarum* strain JCM 1149) were selected for further studies.

5.4 Discussion

The purpose of this study was the assessment of the biodiversity of LAB strains from sourdough collection using a molecular method. The potential influence of isolated LAB from sourdoughs was also investigated; some of them had high antimicrobial activity against food pathogenic microorganisms. Isolated strains are known to be useful as a starter culture for preparing high quality sourdough products, and also to improve the quality and shelf life of the bread products.

PCR-DGGE has been applied to identify microorganisms in sourdough samples. This technique is well-known for reducing the workload and improving the accuracy and efficiency of identification of microorganisms. In recent years, culture- independent methods have been developed to reduce the limitation of conventional cultivation techniques for analysis of microbial communities in fermented foods (Ampe *et al*., 1999; Ercolini, 2004). There is wide range of DNA-based methods that have been used for identification and typing of LAB strains (Temmerman *et al*., 2004). The PCR-DGGE technique is a genetic fingerprinting technique that examines the microbial diversity based upon electrophoresis of PCR-amplified 16S rDNA fragments with gels containing a linear gradient of DNA denaturants (Muyzer *et al*., 1993). The PCR product banding pattern is indicative of the number of bacterial species or assemblages of groupings consisting of species that are present, and thus allows visualisation of the genetic diversity of microbial populations. These amplified fragments may be referred to as PCR products, fragments, or bands. This technique acts as an suitable method for the evaluation of microbial ecosystems. Additionally, it also allows the analysis of large number of samples and detection of shifts in predominant microbial populations. This molecular

fingerprinting technique has been used successfully to describe the variation in bacterial population or microbial community of sourdough (Meroth *et al*., 2004; Randazzo *et al*., 2005).

In this study, homofermentative and heterofermentative species of LAB were found in all the sourdough samples; the results are in agreement with the findings of De Vuyst and Neyssens (2005) and De Vuyst and Vancanneyt (2007) who reported that both of them were found on the natural sourdoughs, whereas, it could be noticed in the previous study that the dominant group of LAB are heterofermentative LAB in sourdough especially when the sourdough is traditionally prepared, as confirmed by De Vuyst *et al*. (2002) and Corsetti *et al*. (2003). Gene sequencing of the results allowed identification of bacterial populations on sourdoughs, showing a predominance of the genus *Lactobacillus* in agreement with the findings of Savic *et al*. (2013) which was also related to diversity and richness indices. During sourdough fermentation, a selection of microbial population with specific nutrient requirements and growth conditions occurs; lactobacilli, among all the bacteria; inhabitants of sourdoughs, are highly adapted to the environmental conditions (temperature, pH, acidity, antimicrobial products, etc.) of sourdough (Vera *et al*., 2009). For this reason, lactobacilli represent the dominant microbial group and commonly occur with the highest concentration, especially in mature sourdoughs. A standard approach to obtain a first preliminary view of the taxonomic diversity, among a set of unknown isolates recovered from a sourdough ecosystem, has long relied on the use of 16S rRNA genes by sequence-based analysis approaches (De Vuyst *et al*., 2002; Meroth *et al*., 2004). According to literature, *Lb. plantarum* is a typical LAB found in wheat and spelt sourdough (Van der Meulen *et al*., 2007)

and is also frequently present in other sourdough ecosystems (De Vuyst and Vancanneyt, 2007). *Lb. sanfranciscensis* was detected from French sourdough but not predominantly (Ferchichi *et al*., 2007; Robert *et al*., 2009) as the same result found from some of the sourdough ecosystem. This could be elucidated by the highly adapted carbohydrate metabolism during cereal fermentations (Kleerebezem *et al*., 2003). The differences in microbial diversity and richness between sourdough samples might be due to collecting various sourdough samples and from different places. Microbial interactions, type of flour, low and variable availability of nutrients, environmental stresses during processing and changes in the technology can be some of the factors which affect the biochemical and physiological responses of LAB in sourdough (Şimşek *et al*., 2006; Hüfner *et al*., 2008; Serrazanetti *et al*., 2009).

The results of this study show that the sourdough samples had different pH and TA values. The pH values varied between (3.46 and 4.36) and TA values varied between (0.67 to 1.05 mg/100mg). SD6 and SD8 had the lowest pH value and highest TA value in comparison to the other sourdough samples. Thirteen sourdoughs had pH values of less than 4.0. The results are in agreement with Minervini *et al*. (2012), who reported that the pH values of thirteen sourdough samples from nineteen had pH under 4.0; this might be due to the microbial action in sourdoughs producing metabolite compounds including lactic acid. The pH of sourdoughs varies according to kind of starter culture, nature of the flour in particular its ash content and nature of the process used, which has an effect on acidification as well (Clarke *et al*., 2002; Clarke and Arendt, 2005). In spontaneous sourdough fermentation, the initial pH usually is within the range

of 5-6.2 while during the fermentation, it reaches approximately 3.5 to 4.2 (Corsetti *et al*., 2001).

Based on the 16S rDNA sequences, all isolated LAB (32) from sourdoughs belonged to the family *Lactobacillaceae*, where more of them are *Lb. plantarum*, *Lb. brevis* and *Lb. rossiae*. However, some others were isolated from sourdoughs such as *Lb. hammesii*, *Lb. parabrevis*, *Lb. farciminis* and *Lb. senmaizukei*. The previous studies showed that the strains of *Lb. plantarum*, *Lb. fermentum*, *Lb. brevis*, *Lb. rossiae*, and *Lb. paraplantarum* were dominant in some of the sourdough ecosystems (Van der Meulen *et al*., 2007). The isolation of more *Lb. plantarum* and *Lb. brevis* groups from the sourdough samples might be due to the dominant group of the sourdough which were assigned to the plant-associated (De Vuyst *et al*., 2014; Zheng *et al*., 2015).

Based on the results, some of the 32 LAB strains presented amylolytic (10) and proteolytic (12) activities. Additionally, from six LAB strains of the high acid group, four and five of them had amylolytic and proteolytic activities respectively. The enzyme activities might be due to a decrease in pH and increase in acid production. A similar reason was reported by Galle (2013) who demonstrated that low pH and acidification would affect the sourdough characteristics including changes in enzyme activity. Arendt *et al*. (2007) also reported that the decrease in pH linked with acid production and caused an increase in the protease and amylase activity of the flour. Good sugar fermenting strains also happened to be hydrolytic enzyme producers, when tested in absence of sugar. Corsetti *et al*. (1998) found that *Lactobacillus plantarum* DC400 and *Lactobacillus sanfrancisco* CB1 were the most proteolytic and amylolytic strains studied. Thiele *et al*. (2002) reported that proteolysis activity increased in

doughs at pH 4 and an acidic range in comparison to non-acidified systems. The proteolytic activity of wheat sourdough depends on the microbial starter and the processing conditions as reported by Katina (2005), who showed that wheat sours, the extraction rate of flour and the fermentation temperature have been found to be the main factors that can have a positive impact on the level of free amino acids, and accumulation of hydrophobic and basic amino acids.

The antibacterial activities of LAB isolates were tested against some food pathogenic strains using agar well diffusion and agar spot methods, the results show that Lb1, Lb2, Lb11, Lb12 and Lb14 strains had higher antibacterial activities against pathogenic strains than the other strains of LAB. The inhibitory action of LAB could be due to the production of antimicrobial compounds based predominantly on organic acids (lactic acid and acetic acid) and bacteriocins. Also hydrogen peroxide and diacetyl have an inhibitory activity which can restrict the growth of potential pathogenic and spoilage microorganisms as discussed in the literature review (Lavermicocca *et al*., 2000; Gerez *et al*., 2008). Şimşek *et al*. (2006) reported that LAB isolated from sourdough samples had different antimicrobial activities against food pathogenic and spoilage microorganisms using agar spot and agar well diffusion methods, which showed that more than one *Lb. plantarum* has a good inhibitory activity against bacterial strains. A *Lb. brevis* strain isolated from Egyptian dairy products had good antimicrobial activity against *Staphylococcus aureus*, *E. coli* and *Salmonella typhi* as reported by Rushdy and Gomaa (2013). Yateem *et al*. (2008) reported that *Lb. plantarum* had a higher inhibitory activity against *E. coli* and *Salmonella* sp. than other LAB strains such as *Lb. pentosus and Lc. lacts* subsp. *lactis*

using an *in-vitro* method. The knowledge about sourdough microflora is useful for selecting LAB strains as starter cultures for sourdough fermentation.

5.5 Conclusion

This study aimed to investigate the bacterial biodiversity of starter cultures and isolates from spontaneously fermented sourdough made with different types of flour. This helps to explore sourdough microbiota, which is useful for selecting LAB strains as starter cultures. Safety, high antimicrobial activity and technological efficacy have to be considered when selecting strains for the food fermentations, to safeguard typical local production which could be better exploited in technological processes by producing a better quality of sourdough fermentation. PCR-DGGE DNA fingerprinting revealed that *Lactobacillus* was the predominant genus in the studied sourdoughs. Thirty-two strains of LAB were isolated and identified using physiological and biochemical tests and further PCR fingerprinting analysis. *Lb. plantarum* and *Lb. brevis* strains accounted for 69% of the 32 isolates. The findings of this study confirm that some of isolated LAB show potential as starter cultures based on acidification capacity, amylolytic and proteolytic activities and antimicrobial activity against food pathogenic microorganisms. However, more studies are needed to apply these strains to the fermented sourdough. There is also potential for fermented intermediary products to be added to the bread formulations to increase the safety, quality, texture, delay staling and increase the shelf life of bread.

CHAPTER SIX

Impact of sourdough fermented with *Lactobacillus plantarum* on the quality and shelf life of sourdough bread

6.1 Introduction

Bread can be counted as one of the earliest of all processed foods, it is certainly known to be one of the first foods to be produced on a large scale (Wood, 1996). Bread in many forms and shapes can be measured as a staple food and it is generally considered as a perishable product. Bread's shelf life is limited by two main factors: staling and microbial action including fungi spoilage and ropiness (Katina, 2005; Arendt *et al*., 2007). Bread is a product consisting of two distinctly different parts: crust and crumb. Varieties of bread can be different according to the in size, shape, colour, texture, and flavour (Hui *et al*., 2007).

Sourdough is an important modern fermentation of cereal flours and water based upon an earlier spontaneous process (Vogel *et al*., 1999; Hammes *et al*., 2005). The sourdough microflora is dominated by LAB that cooperate with yeast and can play a key role in the fermentation of bread dough (Gobbetti, 1998; De Vuyst and Neysens, 2005; Hammes *et al*., 2005; Chavan and Chavan, 2011). The presence of LAB in sourdough results in a sour taste in the end product. At the same time yeast fermentation occurs which leads to the dough expansion. Sourdough fermentations are characterised by the combined activity of LAB and yeasts (Vrancken *et al*., 2010). The aim of utilizing sourdough in bread production is to use microbes as leavening agents which, while using little or no baker's yeasts can cause improvements of dough properties, due to its useful impacts on the flavour, texture and taste of the final product. Moreover, it can

improve a product's nutritional value and extend shelf life of the bread product (Hansen, 2012; De Vuyst and Neysens, 2005; Arendt *et al*., 2007; Dal Bello *et al*., 2007; Nawaz *et al*., 2007; Plessas *et al*., 2011; Gobbetti *et al*., 2014).

Sourdough bread is traditional bread with a natural flavour developed by LAB (mostly *Lactobacillus* genus) and yeast (commercially *Saccharomyces cerevisiae*). The taste and flavour of bread can be improved by the optimal use of sourdough (Seibel and Brummer, 1991). The flavour of sourdough wheat bread is richer and more aromatic than wheat bread, a factor which can be attributed to the long fermentation time of sourdough (Brummer and Lorenz, 1991). Studies on the influence of LAB on the aroma of wheat bread revealed a positive influence, particularly on the crumb aroma (Hansen and Hansen, 1996). The addition of LAB to sourdough can slow down the staling rate during bread storage (Gül *et al*., 2005). Corsetti *et al*. (1998) found that a high ratio of lactic acid to acetic acid could reduce the staling rate and increase volume expansion. The ratio of lactic acid to acetic acid is also an important factor for final product flavour (Linko *et al*., 1997).

The study aimed to investigate the potential changes to bread properties including texture, colour changes and sensory attributes after treatments, as well as to assess the ability of fermented sourdough used for making bread to restrict the growth of pathogenic and spoilage microorganisms, and also to improve safety and shelf life of bread products.

6.2 Materials and Methods

6.2.1 Preparation of cultures for sourdoughs

Lb. *brevis* ATCC 367 (Lb1), *Lb*. *rossiae* DSM15814 (Lb11) and *Lb*. *plantarum* JCM1149 (Lb14) strains were selected from the previous experiment to be used as starter cultures to ferment sourdough. Each inoculum was prepared from an overnight culture and 1% was inoculated into 10ml MRS broth; the cultures were then incubated overnight at 37ºC in the 5% CO_2 incubator. Cells were harvested by centrifugation, washed twice with sterilised water and re-suspended in 150ml water. Bacterial counts were determined by plating on MRS agar 37ºC for 72h while making the sourdough (Rosenquist and Hansen, 1998; Katina *et al*., 2002; Menteş *et al*., 2007).

6.2.2 Culture media

Culture media (*Bacillus cereus* selective agar base, MRS, nutrient agar, plate count agar, potato dextrose agar and yeast extract glucose chloramphenicol agar) were prepared as in Section 2.3.

6.2.3 Preparation of sourdough fermented with starter culture

Sourdoughs were prepared by gently mixing 150g of strong white flour with 150g lukewarm water (30ºC), containing fresh cells of LAB suspension (Lb1, Lb11 and Lb14) at the level of 10^7 CFU/g of dough from each LAB strain alone and mixed cultures. The dough weight was 300g as shown in Table 6.1. The control group was made without adding bacterial suspension. Sourdough

samples (SD1-SD17) were prepared by mixing 10% of each sourdough separately with strong white flour and water except for the first three sourdoughs, prepared according to the company, as shown in Table 6.2. Each dough was mixed separately for 10min until it was the correct consistency. After that, the doughs were covered, then incubated at 30°C for 24h to be fermented, and then back slopping was used for further sourdough fermentation. 150g of fermented sourdough in the first day of fermentation was mixed with 75g of flour and 75ml of water. Then the fermentation was repeated under the same conditions until five days of back slopping. Three replicates were made for the experiment.

Table 6.1: Design of the sourdough fermentation batches

Culture	Treatment	Microorganisms	Inoculum volume in g of water	Ingredients	Dough weight
Single culture	SIN1	*Lb brevis* Lb1	150	Strong white flour (150g) + water (150 g)	300
	SIN2	*Lb. rossiae* Lb11	150		300
	SIN3	*Lb. plantarum* Lb14	150		300
Mixed culture	MIX1	*Lb. brevis* Lb1 + *Lb. rossiae* Lb11	75/75	Strong white flour (150g) + water (150 g)	300
	MIX2	*Lb. rossiae* Lb11 + *Lb. plantarum* Lb14	75/75		300
	MIX3	*Lb. brevis* Lb1 + *Lb. plantarum* Lb14	75/75		300
	MIX4	*Lb. brevis* Lb1 + *Lb. rossiae* Lb11 + *Lb. plantarum* Lb14	50/50/50		300
Control	COND	Without culture	-	Strong white flour (150g) + water (150 g)	300

Table 6.2: Sourdough fermentation batches with sourdough collection samples (from chapter 5)

Sourdough samples*	Treatment	Sourdough mixture		
		Sourdough samples (g)	White strong flour (g)	Water (g)
SD1	SDP1	Made according to the company		
SD2	SDP2	5	5000	5000
SD3	SDP3			
SD4–SD17	SDP4- SDP17	10	45	45

* Key of the table as Table 5.1

6.2.4 Measuring pH value of sourdough samples

The pH values of sourdough starter before and after fermentation were measured as mentioned in Section 4.2.5.1.

6.2.5 Measuring TA of sourdough samples

The TA values of sourdough starter before and after fermentation were measured as mentioned in Section 4.2.5.2.

6.2.6 Measuring Organic acids of sourdough fermentation

About 0.5±0.01g of fermented sourdough samples were added to 1ml of Milli-Q water. Samples were mixed for 30sec using a vortex mixer and centrifuged at 13000xg for 20min. To 100 µl of supernatant in 400 µl of Milli-Q water (Millipore Corp., Bedford, MA, USA) was added 20µl of 92mmol/L H_2SO_4. The

supernatant was extracted using 1ml polypropylene disposable syringe (Fisher Scientific, BD A-Line, UK) and filtered through Millipore microfilter (0.20µm pore size) (SMI-LabHut Ltd, Gloucester, UK) in to a vial and sealed with a crimp cap (11mm, Ruber/PTFE, Fisher Scientific, Loughborough, UK). The organic acid (lactic acid, acetic acid and propionic acid) composition of the fermented sourdough samples was analysed in triplicate using High Performance Liquid Chromatography (HPLC) (Gynkotek, Dionex Corp., Sunnyvale, CA, USA) according to the method of Niven *et al*. (2004).

All data obtained were processed using Chromeleon® 7.1 Chromatography Data System Software (Dionex Softron GmbH, Germering, Germany). A calibration curve for each (lactic acid, acetic acid and propionic acid) was obtained from six different concentrations (0.2µmol, 2µmol, 20µmol, 200µmol, 2mmol and 20mmol) of the standards' stock solutions.

6.2.7 Microbial count of sourdough

The aerobic plate count (APC), cell counts of LAB, and yeasts in sourdough fermentation samples were determined by the viable cell count method on BHI agar, MRS agar and yeast extract glucose chloramphenicol agar respectively.

6.2.8 Sourdough microflora analysis

The sourdough samples, after fermentation as described in Section 6.2.3, were put into a sterile 1.5ml Eppendorf tube. The samples were stored at −20°C until DNA extraction. All procedures were as described in Section 5.2.2, including

DNA extraction, polymerase chain reaction (PCR) followed by agarose gel electrophoresis (AGE), and denaturant grade gel electrophoresis (DGGE) analysis and lastly gene sequences. Selected bands (OTU) of DGGE gel were aseptically separated and sequenced according to whether the band represented many groups or was a unique band for particular groups. BLAST at NCBI was used to confirm the species of the bacteria. The phylogenetic tree of the 16S rRNA gene sequences was constructed in MEGA 6 using the neighbour joining method as mentioned in Section 5.2.2.6.

According to the results of pH, acidity, presence of organic acids and microbial growth, the sourdough that was made with single culture Lb14 (*Lactobacillus plantarum* strain JCM 1149) from SIN3 had good results and was selected for further studies.

6.2.9 Preparation of bread and baking

Breads were prepared according to the recipe of Tovar *et al*. (1992) and Tudorica (2004) using batches of 500g basic mixture dough 60% of white strong flour, 40% water and the active sourdough with *Lb. plantarum* JCM1149 (SIN3) were added using 9% and 18% on the base of flour and water. Dried active yeast (0.6g/100g of the mixture), salt (0.6g/100g of the mixture) and oil (0.3g/100g of the mixture) were added as a portion for each dough. The control bread was made without sourdough addition. The ingredients were mixed for 5min in an electric high speed mixer - Robot Coupe R4 (Robot Coupe Ltd, UK). After hand moulding, the dough pieces were put into tins and proofed at different temperatures and different fermentation times in two groups as shown

in Table 6.3. The first group of doughs were left for 3h at 30°C (fast fermentation) while the other group were left for 18h at 20°C (slow fermentation) to ferment. The bread was baked in an oven (Challenger, Garland, A Welbilt Company, Model E9E, USA) at 220°C for 20min. Afterwards, the bread was left to cool at room temperature and they were placed in polyethylene bags. Baking tests were run in triplicates.

Table 6.3: The experiment design and treatment formulations of sourdough bread (%)

Ingredients	Fast fermentation 3 h at 30°C			Slow fermentation 18 h at 20°C		
	FFCON*	FFLSD	FFHSD	SFCON	SFLSD	SFHSD
Strong white bread flour	60	55.5	51	60	55.5	51
Water	40	35.5	31	40	35.5	31
Sourdough	0	9	18	0	9	18

Dried active yeast (0.6g/100g of the mixture), salt (0.6g/100g of the mixture), oil (0.3g/100g of the mixture)

* FFCON, Fast fermentation control bread
FFLSD, Fast fermentation low level sourdough bread
FFHSD, Fast fermentation high level sourdough bread
SFCON, Slow fermentation control bread
SFLSD, Slow fermentation low level sourdough bread
SFHSD, Slow fermentation high level sourdough bread

6.2.10 Physical and chemical properties of bread samples

6.2.10.1 pH and TA values of bread samples

The pH and TA values of dough before baking and bread at storage period at room temperature were measured as mentioned in Section 4.2.5.1 and 4.2.5.2 respectively.

6.2.10.2 Moisture of bread samples

Moisture content was measured according to AACC 44.01 (2000). The bread samples were weighed and dried at 103±2°C with a fan assisted oven (Genlab Ltd., UK). Samples were taken out from the oven at definite time intervals and weighed after cooling in a desiccator. This action was continued until achieving a fixed weight. Finally, moisture content was calculated using the equation below. Three readings per bread sample were run.

$$\text{Moisture \%} = \frac{\text{Sample weight (g)} - \text{Dry weight of the sample (g)}}{\text{Sample weight (g)}} \times 100$$

6.2.10.3 Loaf volume of bread samples

The loaf volume of the sourdough bread was measured with a standard method rapeseed displacement method. A container of known size was filled with rapeseed and then samples placed in the container followed by rapeseed. The extra rapeseed which is no longer able to fill the container is deemed as loaf volume. Three readings per sample batch were run.

6.2.10.4 Water activity of bread samples

Water activity (Aw) measurement was determined in triplicate on each bread sample. The determination was mentioned in Section 4.2.5.3.

6.2.10.5 Instrumental evaluation of bread quality

Texture profile analyser (TPA) from Texture analyser (TA-TX2-Stable Micro System, UK) was used to evaluate sourdough bread quality parameters (hardness, springiness and chewiness). 2cm height of bread crumb was taken from the bread and tested. The settings were: pre-test speed: 1.0mm/s; test speed: 1mm/s; post-test speed; 1mm/s; distance: 40%, auto 5g trigger force, with 12.5mm cylinder probe with radius (P/12.5R). Texture analysis was measured every two days during storage of bread samples. Six replicates per each sample batch were assessed.

6.2.10.6 Colour of bread samples

Bread crust and crumb colour *L**, *a** and *b** were measured as described in Section 2.7.4.

6.2.10.7 Image analysis of bread samples

Breads were cut into two pieces then bread crumbs were photographed by a digital camera (Sony Cyber shot DSC-HX30V, Japan) in a constant place with white light. The image J software (version 1.49) was used for analysing the porosity of bread crumb which was between two phases (pores and solid part). The photographed colour images were first converted to grey scale. Using bars of known length, pixel values were converted into distance units. The largest possible rectangular cross-section with the same size of the each bread was cropped. After adjusting the threshold, the total pore area was measured using the software. However, height of bread samples was measured via image J. Three replicates were measured for each treatment.

6.2.11 Microbial shelf life of breads

The microbial shelf life of bread samples is described in Section 2.7.5.

6.2.12 Sensory evaluation

Bread samples were subjected to sensory evaluation by 33 panellists the day after baking, which is described in Section 2.8.

6.2.13 Statistical analysis

All data were analysed statistically as detailed in Section 2.9 and 5.2.10.

6.3 Results

6.3.1 pH and TA values of sourdough samples

The pH and TA values of the sourdoughs were tested before and after fermentation from day four of sourdough refreshment for day five at 30ºC as shown in the Table 6.4. There were no significant differences (P>0.05) in pH values among sourdoughs before fermentation, where the pH values varied from 5.81 to 5.88. After 24h of fermentation at day five, there were significant differences (P<0.05) in pH values between some sourdoughs. The pH values varied from 3.34 to 5.02, where the lower pH values belong to SIN3 and the higher belong to COND. Sixteen sourdough samples had pH values of less than 4.0 after 5 days of back-slopping at 30ºC.

There were significant differences (P<0.05) in TA values between some sourdough samples before and after the fermentation period. The TA values of the sourdoughs varied from 0.19 to 0.36mg/100mg before fermentation of the sourdoughs, where the lower pH value belongs to SDP14 and the higher belongs to SDP8, while the TA values varied from 0.67 to 1.53mg/100mg after fermenting the sourdoughs. SIN3 had a high TA value (1.53mg/100mg) and significant differences in comparison with the other sourdoughs after the fermentation period. However, COND and SDP17 had lower TA values after sourdoughs fermented at 30ºC for 24h at the fifth day of refreshment.

Table 6.4: pH and TA value* measurements during sourdough fermentation before and after from day four of sourdough refreshment for day 5 at 30ºC

Treatments**	pH values		TA values (mg/100mg)	
	0h/day 4	24h/ day 5	0h/day 4	24h/ day 5
SIN1	5.88±0.02	3.46±0.01 ^{n}	0.27±0.02 ^{efg}	1.31±0.02 ^{bc}
SIN2	5.84±0.01	3.42±0.01 ^{n}	0.32±0.00 ^{bc}	1.4±0.01 ^{ab}
SIN3	5.88±0.02	3.34±0.01 ^{o}	0.32±0.01 ^{bc}	1.53±0.07 ^{a}
MIX1	5.82±0.01	3.42±0.01 ^{n}	0.28±0.01 ^{def}	1.39±0.1 ^{b}
MIX2	5.81±0.02	3.59±0.01 ^{lm}	0.31±0.01 ^{bcd}	1.33±0.05 ^{bc}
MIX3	5.87±0.01	3.55±0.01 ^{m}	0.33±0.01 ^{bc}	1.34±0.01 ^{bc}
MIX4	5.81±0.01	3.61±0.01 ^{l}	0.31±0.01 ^{bcd}	1.21±0.01 ^{cd}
COND	5.85±0.02	5.02±0.02 ^{a}	0.23±0.07 ^{hijk}	0.67±0.02 ^{k}
SDP1	5.85±0.04	3.82±0.02 ^{ij}	0.26±0.01 ^{fgh}	0.79±0.02 ^{ijk}
SDP2	5.86±0.04	3.78±0.01 ^{j}	0.29±0.01 ^{def}	0.84±0.02 ^{hij}
SDP3	5.85±0.02	3.70±0.02 ^{k}	0.30±0.01 ^{cde}	0.92±0.02 ^{fgh}
SDP4	5.85±0.02	3.87±0.02 ^{i}	0.28±0.01 ^{def}	0.79±0.02 ^{ijk}
SDP5	5.82±0.01	4.70±0.03 ^{d}	0.22±0.01 ^{ijkl}	0.68±0.01 ^{k}
SDP6	5.82±0.01	3.60±0.02 ^{lm}	0.33±0.01 ^{ab}	1.14±0.01 ^{de}
SDP7	5.86±0.02	4.83±0.02 ^{c}	0.22±0.01 ^{ijkl}	0.94±0.02 ^{fgh}
SDP8	5.83±0.02	3.67±0.02 ^{k}	0.36±0.01 ^{a}	1.18±0.01 ^{d}
SDP9	5.81±0.04	4.80±0.02 ^{c}	0.20±0.01 ^{kl}	0.71±0.02 ^{jk}
SDP10	5.85±0.04	3.86±0.02 ^{i}	0.24±0.02 ^{hij}	0.86±0.01 ^{ghi}
SDP11	5.83±0.02	4.95±0.03 ^{b}	0.22±0.01 ^{ijkl}	0.77±0.01 ^{ijk}
SDP12	5.86±0.02	4.31±0.02 ^{f}	0.25±0.02 ^{ghi}	1.03±0.01 ^{ef}
SDP13	5.83±0.09	3.96±0.02 ^{h}	0.33±0.01 ^{bc}	1.00±0.01 ^{fg}
SDP14	5.81±0.02	4.40±0.02 ^{e}	0.19±0.02 ^{l}	0.79±0.02 ^{ijk}
SDP15	5.85±0.02	4.14±0.02 ^{g}	0.26±0.01 ^{fgh}	0.85±0.01 ^{hi}
SDP16	5.82±0.01	3.97±0.02 ^{h}	0.22±0.01 ^{ijkl}	0.77±0.01 ^{ijk}
SDP17	5.83±0.01	4.44±0.03 ^{e}	0.21±0.01 ^{jkl}	0.67±0.01 ^{k}

* Mean values from three replicates ± standard deviations. $^{a-l}$ Means in each column with different superscripts are significant different (P<0.05) .

** Key of the table as Table 6.1 and 6.2

6.3.2 Microbial growth of sourdough samples

Growth of microorganisms (LAB, APC and yeasts) are shown in Table 6.5. The range of LAB growth in prepared sourdough fermentation after 5 days of back-slopping at 30ºC varied between 5.1 and 9.86 Log_{10}CFU/ml. The number of LAB in SIN3 was significantly (P<0.05) higher than the number of LAB in other sourdough samples. APC and yeast count in sourdough fermentation samples after five days of back-slopping at 37ºC (for APC) and 25ºC (for yeast) ranged from 4.70 to 6.6 Log_{10}CFU/ml and 3.54 to 4.32 Log_{10}CFU/ml respectively. The number of APC was significantly lower in sourdoughs SIN2, SIN3 and MIX2 compared to the other sourdoughs. The yeast count was significantly higher in SDP6 compared to the other sourdoughs. There was no yeast growth in some fermented sourdoughs (SDP9, SDP11, SDP14 and SDP15). Consequently, the number of LAB, APC and yeast in COND was 5.33, 7.23 and 3.5 Log_{10}CFU/ml respectively.

Table 6.5: Growth of LAB, APC and yeasts* in the fermented sourdoughs preparation after 5 days of back-slopping at 30ºC, 37ºC, and 25ºC respectively

Treatments**	Microorganisms (Log_{10}CFU/ml)		
	LAB	APC	yeasts
SIN1	9.10±0.03 ^{d}	4.94±0.02 ^{mn}	4.12±0.01 ^{d}
SIN2	9.59±0.15 ^{b}	4.79±0.01 ^{op}	4.19±0.01 ^{bc}
SIN3	9.86±0.08 ^{a}	4.73±0.01 ^{pq}	4.16±0.01 ^{cd}
MIX1	9.25±0.03 ^{cd}	4.99±0.01 ^{lm}	3.87±0.02 ^{g}
MIX2	9.44±0.02 ^{bc}	4.70±0.01 ^{q}	3.95±0.01 ^{f}
MIX3	9.41±0.02 ^{bc}	4.86±0.01 ^{no}	4.03±0.01 ^{e}
MIX4	9.20±0.07 ^{cd}	5.06±0.04 ^{kl}	3.86±0.03 ^{g}
COND	5.33±0.02 ^{no}	7.23±0.04 ^{a}	3.50±0.02 ^{k}
SDP1	7.43±0.03 ^{g}	5.10±0.04 ^{k}	3.54±0.02 ^{jk}
SDP2	6.10±0.08 ^{jk}	5.40±0.02 ^{hi}	4.04±0.03 ^{e}
SDP3	6.71±0.09 ^{h}	5.69±0.06 ^{g}	3.85±0.02 ^{g}
SDP4	7.36±0.14 ^{g}	5.44±0.01 ^{h}	4.20±0.01 ^{bc}
SDP5	6.42±0.04 ^{i}	6.17±0.03 ^{f}	3.96±0.02 ^{f}
SDP6	8.47±0.19 ^{f}	5.09±0.02 ^{k}	4.32±0.01 ^{a}
SDP7	6.96±0.07 ^{h}	5.34±0.02 ^{i}	4.03±0.03 ^{e}
SDP8	8.80±0.08 ^{e}	4.96±0.01 ^{m}	4.24±0.02 ^{b}
SDP9	5.32±0.06 ^{no}	5.24±0.05 ^{j}	0.00±0.00 ^{l}
SDP10	6.19±0.06 ^{ijk}	5.35±0.02 ^{i}	3.77±0.02 ^{h}
SDP11	5.41±0.05 ^{n}	6.38±0.01 ^{de}	0.00±0.00 ^{l}
SDP12	5.68±0.16 ^{m}	5.37±0.02 ^{hi}	3.51±0.02 ^{k}
SDP13	6.27±0.05 ^{ij}	6.13±0.01 ^{f}	3.59±0.02 ^{ij}
SDP14	5.95±0.04 ^{kl}	6.32±0.01 ^{e}	0.00±0.00 ^{l}
SDP15	6.36±0.03 ^{i}	6.42±0.01 ^{cd}	0.00±0.00 ^{l}
SDP16	5.75±0.05 ^{lm}	6.47±0.02 ^{c}	3.63±0.03 ^{i}
SDP17	5.10±0.02 ^{o}	6.66±0.02 ^{b}	3.51±0.04 ^{k}

* Mean values from three replicates ± standard deviations. $^{a-q}$ Means in each column with different superscripts are significant different (P<0.05) .

** Key of the table as Table 6.1 and 6.2

6.3.3 Organic acid concentrations of sourdough samples

The results of the organic acids in the fermented sourdough samples are shown in the Table 6.6. Lactic acid, acetic acid and propionic acid production were in the ranges 1.33 -139.78mmol/L, 4.97 - 29.03mmol/L and 1.67 - 12.87mmol/L respectively. There were no significant differences (P>0.05) in the lactic acid production in the three sourdoughs (SIN3, SDP3 and SDP8). There were also no significant differences (P>0.05) among the sourdoughs made by single and mixture cultures of LAB. Sourdough fermented with single and mixture cultures of LAB could produce higher amount of lactic acid in comparison with the other sourdoughs except SDP3 and SDP8. Sourdough preparation SDP15 and SDP16 could produce higher amounts of acetic acid and low amounts of lactic acid. High amounts of propionic acid were produced by the SDP15 which was significantly different (P<0.05) to the other sourdough production. Some of the sourdough samples had not produced propionic acid, as shown in Table 6.6. Through sourdough fermentation, LAB produced organic acids which directly related to a decrease in pH level (high organic production decrease level of pH) which is clearly shown in Table 6.4 and 6.6.

Table 6.6: Lactic acid, acetic acid and propionic acid* of fermented sourdoughs after 5 days of back-slopping at 30ºC

Treatments**	Organic acids (mmol/L)		
	Lactic acid	Acetic acid	Propionic acid
SIN1	118.61±6.11 bcd	5.93±2.12 j	2.33±0.58 cd
SIN2	123.12±1.30 bcd	8.33±0.21 ghij	0
SIN3	131.36±1.74 ab	7.13±0.06 hij	2.00±0.17 d
MIX1	122.64±7.20 bcd	8.13±0.5 ghij	1.67±0.16 d
MIX2	124.87±4.51 bc	6.93±1.44 ij	0
MIX3	125.66±1.55 bc	7.10±0.72 hij	2.80±0.1 cd
MIX4	125.69±6.52 bc	7.73±0.45 hij	2.23±0.06 cd
COND	23.70±2.92 h	12.83±2.61 fghi	0
SDP1	51.22±2.81 g	8.07±0.56 ghij	0
SDP2	115.63±6.44 cd	6.17±0.15 j	0
SDP3	127.89±11.04 abc	20.43±2.4 bc	2.17±0.06 cd
SDP4	3.11±0.9 i	13.07±1.95 efgh	0
SDP5	2.06±0.58 i	19.07±2.91 cde	6.43±0.25 b
SDP6	95.60±9.05 e	12.83±1.06 fghi	0
SDP7	1.33±0.5 i	4.97±1.77 j	6.57±1.60 b
SDP8	139.78±5.83 a	18.00±2.14 cdef	2.97±0.12 cd
SDP9	2.76±0.19 i	7.20±2.46 hij	0
SDP10	110.70±0.72 d	13.80±2.39 defg	0
SDP11	11.23±3.77 hi	22.27±3.45 bc	0
SDP12	7.03±1.53 i	19.27±1.00 cd	0
SDP13	74.06±1.85 f	18.63±1.99 cdef	0
SDP14	5.97±1.13 i	21.10±1.73 bc	3.70±0.62 c
SDP15	9.56±0.64 i	25.70±1.02 ab	12.87±1.82 a
SDP16	8.00±0.15 i	29.03±3.63 a	0
SDP17	7.92±2.34 i	17.93±1.72 cdef	0

* Mean values from three replicates ± standard deviations. [a-j] Means in each column with different superscripts are significant different (P<0.05) .

** Key of the table as Table 6.1 and 6.2

6.3.4 PCR-DGGE of sourdough samples

Figure 6.1 shows the PCR–DGGE bacterial profiles of the sourdough samples. Many different bands are shown in the DGGE image and the gel bands which were considered to be the operative taxonomy unit (OTU) in each sample.

The similarity of bacterial populations within and between the sourdough samples were measured by using half matrix similarity (%) of sourdoughs DGGE fingerprints as shown in Table 6.7; non-metric multidimensional scaling (MDS) and cluster analysis of DGGE fingerprints are shown in Figure 6.2. There was about 40% similarity of bacterial population between all the sourdough samples. There was more than 80% similarity between all sourdough samples except the control dough (CONA, CONB and CONC).

The diversity and richness indexes were used to display the microbial population's diversity and richness in the sourdough samples as shown in table 6.8. The diversity and richness of the bacterial community based on the PCR-DGGE DNA fingerprinting of sourdough samples indicated that; there were significant differences (P<0.05) in diversity and richness among sourdough samples. The diversity varied between 6.34 and 7.74, and richness between 3.05 and 3.26. The diversity and richness of COND was significantly higher than the other sourdough samples, reaching 7.74 and 3.26 respectively. There were no significant differences in the diversity between single and mixed culture sourdoughs, except MIX4 in which all three LAB mixed to make a sourdough. Also, there were no significant differences in the richness between SIN3, MIX1, MIX2, MIX3 and MIX4.

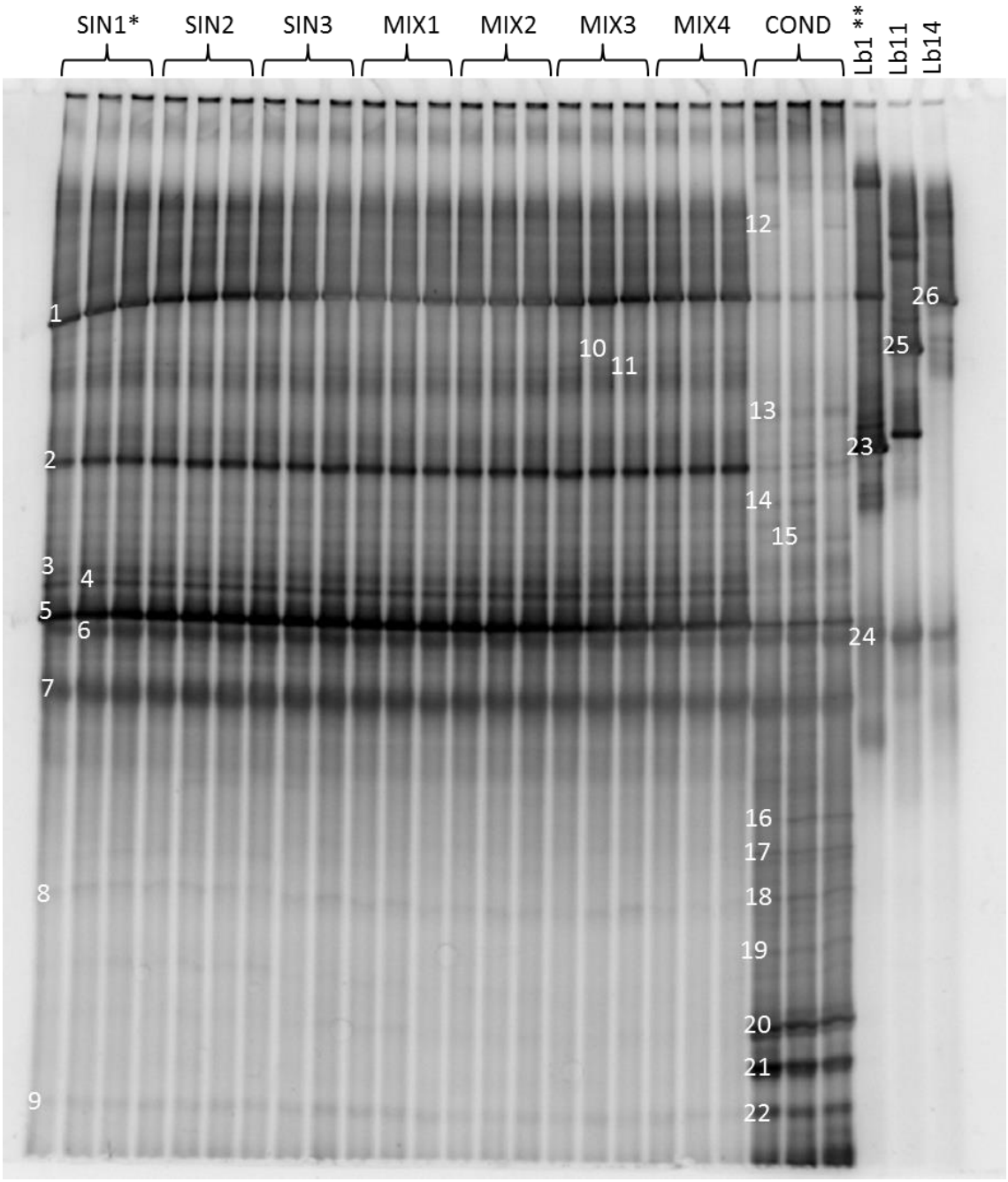


Figure 6.1: DGGE fingerprints of sourdough samples. Numbers of bands (operative taxonomy units (OTUs)) in each sample relates to diversity richness

* Key of the figure as Table 6.1

** (Lb1) *Lb. brevis* ATCC 367, (Lb11) *Lb. rossiae* DSM15814 and (Lb14) *Lb. plantarum* JCM1149

Table 6.7: The half matrix similarity of bacterial population of DGGE fingerprints of sourdough samples which they were made with *Lb. brives*, *Lb. rossie* and *Lb. plantarum* separately and mixed together

Samples*	SIN1 A	SIN1 B	SIN1 C	SIN2 A	SIN2 B	SIN2 C	SIN3 A	SIN3 B	SIN3 C	MIX 1A	MIX 1B	MIX 1C	MIX 2A	MIX 2B	MIX 2C	MIX 3A	MIX 3B	MIX 3C	MIX 4A	MIX 4B	MIX 4C	CON A	CON B	CON C
SIN1A	100																							
SIN1B	100	100																						
SIN1C	100	100	100																					
SIN2A	96	100	96	100																				
SIN2B	96	100	96	100	100																			
SIN2C	96	96	96	100	100	100																		
SIN3A	95	95	95	95	100	95	100																	
SIN3B	95	95	95	95	100	95	100	100																
SIN3C	95	95	95	95	95	95	100	100	100															
MIX1A	95	95	95	95	95	95	100	100	100	100														
MIX1B	95	95	95	95	95	95	100	100	100	100	100													
MIX1C	95	95	95	95	95	95	100	100	100	100	100	100												
MIX2A	95	95	95	95	95	95	100	100	100	100	100	100	100											
MIX2B	95	95	95	95	95	95	100	100	100	100	100	100	100	100										
MIX2C	95	95	95	95	95	95	100	100	100	100	100	100	100	100	100									
MIX3A	95	95	95	95	95	95	100	100	100	100	100	100	100	100	100	100								
MIX3B	95	95	95	95	95	95	100	100	100	100	100	100	100	100	100	100	100							
MIX3C	95	95	95	95	95	95	100	100	100	100	100	100	100	100	100	100	100	100						
MIX4A	93	93	93	93	93	93	98	98	98	98	98	98	98	98	98	98	98	98	100					
MIX4B	93	93	93	93	93	93	98	98	98	98	98	98	98	98	98	98	98	98	100	100				
MIX4C	93	93	93	93	93	93	98	98	98	98	98	98	98	98	98	98	98	98	100	100	100			
CONA	40	40	40	44	44	44	42	42	42	42	42	42	42	42	42	42	42	42	38	38	38	100		
CONB	47	47	47	51	51	51	49	49	49	49	49	49	49	49	49	49	49	49	46	46	46	95	100	
CONC	38	38	38	43	43	43	40	40	40	40	40	40	40	40	40	40	40	40	36	36	36	94	88	100

* Key of the table as table 6.1, A-C refers to replicate number in each treatment

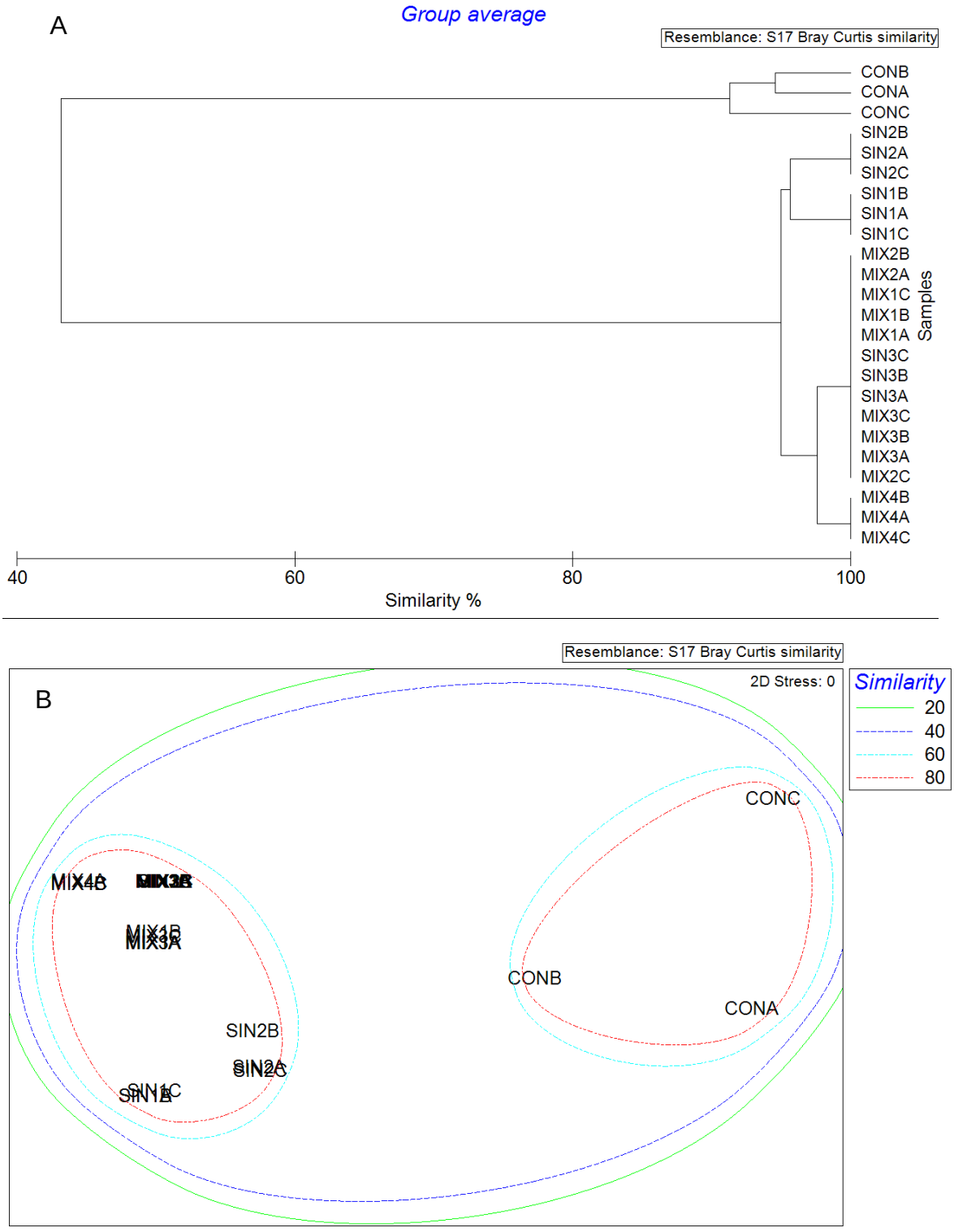


Figure 6.2: (A) Cluster analysis and (B) non-metric multidimensional scaling (MDS) graph based on the PCR-DGGE DNA fingerprints showing similarity (%) of bacterial communities between sourdough samples

* Key of the figures as table 6.1

Table 6.8: Diversity index of bacterial community* in sourdough samples based on the PCR-DGGE DNA fingerprinting

Samples[1]	Band No.	Diversity[2]	Richness[3]
SIN1	23 ^{b}	7.02 ^{b}	3.14 ^{b}
SIN2	23 ^{b}	7.02 ^{b}	3.14 ^{b}
SIN3	21 ^{bc}	6.57 ^{bc}	3.05 ^{c}
MIX1	21 ^{bc}	6.57 ^{bc}	3.05 ^{c}
MIX2	21 ^{bc}	6.57 ^{bc}	3.05 ^{c}
MIX3	21 ^{bc}	6.57 ^{bc}	3.05 ^{c}
MIX4	20 ^{c}	6.34 ^{c}	3.00 ^{c}
COND	26 ^{a}	7.74 ^{a}	3.26 ^{a}

* $^{a-c}$ Means with the different superscript in the same column and age are significantly different ($P<0.05$).
[1] Key of the table as Table 6.1
[2] Diversity: H" = -SUM (pi*Log (pi)).
[3] Richness: d = (S -1)/log (N).

Figure 6.3 shows the phylogenetic relationship among LAB strains that were constructed based on the 16S rDNA sequences using the neighbour joining method. The identification of bands in PCR-DGGE fingerprints of the bacterial population of sourdough samples were selected from the bands in Figure 6.1 for sequencing. Most of the bands that were found are *Lactobacillus* species. The maximum identity of bacterial species was varied between 97 to 99%. There were some uncultured bacteria found in the selected bands; they were discarded due to their maximum identity which were less than 90%. These might be mainly as-yet-uncultured bacteria in the data banks.

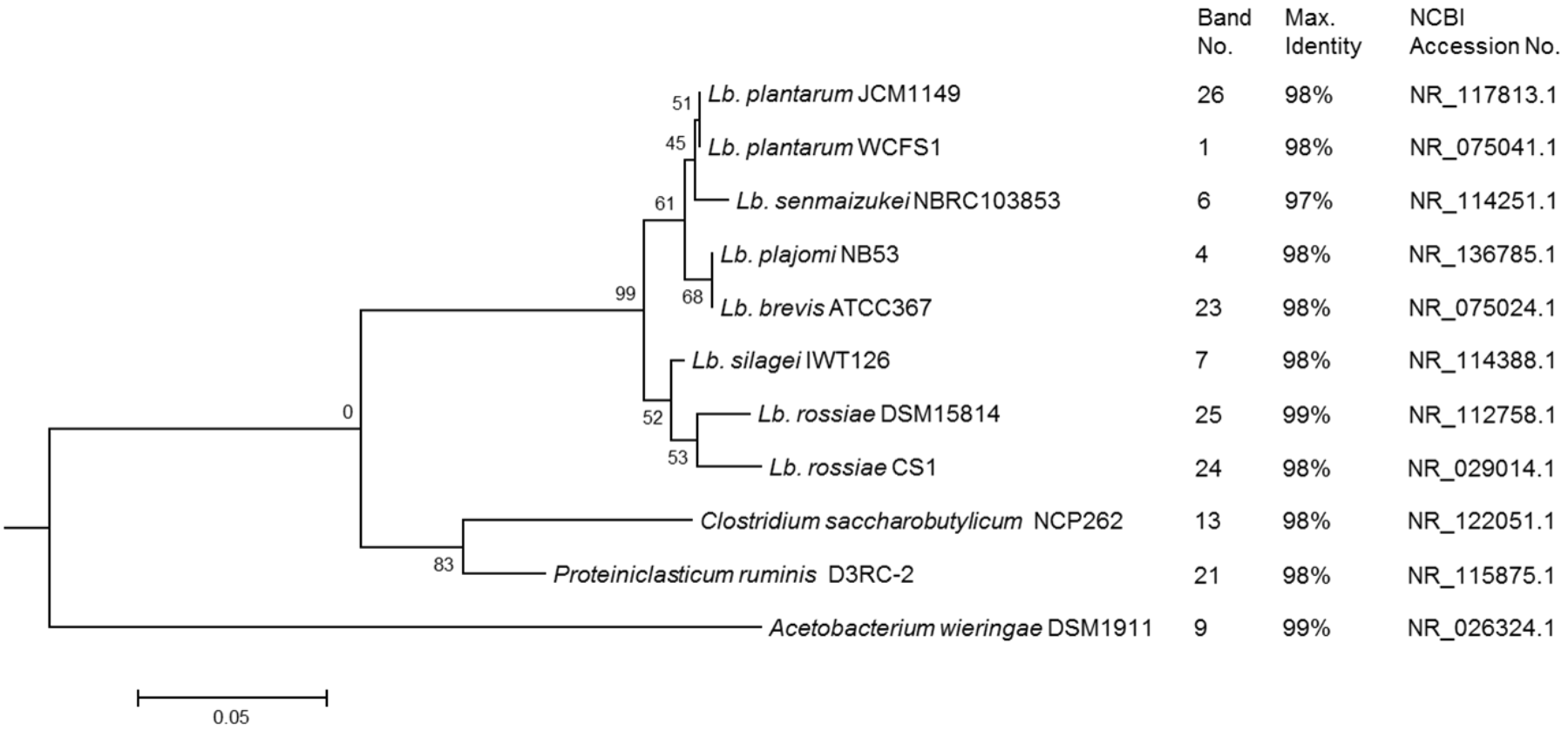


Figure 6.3: Phylogenetic tree showing the relative position of sourdough LAB based on 16S rDNA, using the neighbour joining method. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree, using MEGA 6 software. The scale bar corresponds to 0.05 units of the number of base substitutions per site.

6.3.5 pH and TA values of dough samples before baking

The pH and TA of the dough are important during sourdough fermentation. Before and after the fermentation process of the dough, the pH and TA values were measured as shown in Table 6.9. The results indicated that there were no significant differences (P>0.05) in pH values before starting fermentation, except FFHSD and SFHSD, which might be due to adding more fermented sourdough. However, the TA values were significantly different (P<0.05) before starting fermentation due to the presence of fermented sourdough that was added to the dough at different concentrations. The results indicated that pH and TA values changed significantly (P<0.05) between all treatments after fast fermentation (3h) at 30ºC and slow fermentation (18h) at 20ºC of the doughs.

Table 6.9: pH and TA values* of the doughs before and after fermentation at different temperature and different time interval

Treatment**	Fermentation process	pH		TA (mg/100mg)	
		before	after	before	after
FFCON	3h at 30°C	6.22±0.01 [a]	5.67±0.01 [b]	0.25±0.01 [c]	0.49±0.01 [e]
FFLSD		6.19±0.02 [a]	5.43±0.02 [d]	0.28±0.01 [b]	0.60±0.01 [c]
FFHSD		6.14±0.02 [b]	4.97±0.01 [f]	0.30±0.01 [a]	0.80±0.01 [a]
SFCON	18h at 20°C	6.22±0.01 [a]	5.79±0.02 [a]	0.25±0.01 [c]	0.46±0.01 [f]
SFLSD		6.19±0.02 [a]	5.52±0.01 [c]	0.28±0.01 [b]	0.56±0.01 [d]
SFHSD		6.14±0.02 [b]	5.08±0.02 [e]	0.30±0.01 [a]	0.69±0.01 [b]

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). [a-f] Means in each column with different superscripts are significant different (P<0.05).

** Key of the table as Table 6.3

6.3.6 pH and TA values of bread samples

The pH and TA values of bread samples during the storage period at room temperature are shown in Table 6.10 and 6.11 respectively. On day 0, there were significant differences (P<0.05) in the pH values between treatments, except FFHSD and SFHSD, where pH values ranged from 5.10 to 5.98. There were also significant differences on other days. The pH of the bread with FFHSD was lower than the other treatments. During the storage period over 10 days at room temperature, the pH values were decreased significantly (P<0.05) at all treatments. The TA values ranged from 0.36 to 0.77 on day 0. The TA values of sourdough bread were always higher than control breads. The TA values that were defined significantly increased (P<0.05) during storage over 10 days at room temperature which was due to a decrease in pH values. There were no tests determined on day 8 of storage for the control bread (SFCON) and on day 10 for breads (FFCON, SFCON and SFLSD) due to of microbial growth which was observed on the surface of the breads.

Table 6.10: pH* development over 10 days of storage of sourdough bread at room temperature

Treatment[1]	Fermentation process	Time (days)					
		0	2	4	6	8	10
FFCON	3h at 30°C	5.98±0.01 ^{aA}	5.96±0.01 ^{aB}	5.94±0.01 ^{aC}	5.93±0.01 ^{aC}	5.91±0.01 ^{aD}	Spoiled
FFLSD		5.55±0.01 ^{cA}	5.53±0.02 ^{cAB}	5.52±0.01 ^{cAB}	5.51±0.01 ^{cBC}	5.50±0.01 ^{bBC}	5.49±0.01 ^{aC}
FFHSD		5.11±0.01 ^{eA}	5.10±0.01 ^{eA}	5.09±0.01 ^{eAB}	5.08±0.01 ^{eBC}	5.07±0.01 ^{dC}	5.05±0.01 ^{bD}
SFCON	18h at 20°C	5.93±0.01 ^{bA}	5.92±0.01 ^{bA}	5.89±0.01 ^{bB}	5.88±0.01 ^{bB}	Spoiled[2]	Spoiled
SFLSD		5.50±0.01 ^{dA}	5.48±0.01 ^{dB}	5.46±0.01 ^{dC}	5.44±0.01 ^{dCD}	5.43±0.01 ^{cD}	Spoiled
SFHSD		5.10±0.01 ^{eA}	5.09±0.01 ^{eAB}	5.07±0.01 ^{fBC}	5.06±0.01 ^{eCD}	5.05±0.01 ^{dCD}	5.04±0.01 ^{bD}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-f}$ Means in the same column with different superscripts are significantly different ($P < 0.05$). $^{A-D}$ Means in the same row with different superscripts are significantly different ($P<0.05$).

[1] Key of the table as Table 6.3

[2] Spoiled: Microbial growth was observed on the surface of the breads

Table 6.11: Development of TA* values (mg/100mg) over 10 days of storage of sourdough bread at room temperature

Treatment[1]	Fermentation process	Time (days)					
		0	2	4	6	8	10
FFCON		0.38±0.01 ^{eC}	0.38±0.01 ^{eC}	0.40±0.02 ^{eB}	0.40±0.01 ^{eB}	0.41±0.01 ^{eA}	Spoiled
FFLSD	3h at 30°C	0.58±0.01 ^{cC}	0.59±0.01 ^{cC}	0.59±0.00 ^{cC}	0.59±0.01 ^{cC}	0.61±0.01 ^{cB}	0.63±0.01 ^{cA}
FFHSD		0.77±0.01 ^{aD}	0.78±0.01 ^{aCD}	0.80±0.01 ^{aC}	0.82±0.01 ^{aB}	0.83±0.01 ^{aA}	0.84±0.01 ^{aA}
SFCON		0.36±0.01 ^{eB}	0.37±0.01 ^{eB}	0.39±0.006 ^{eA}	0.40±0.01 ^{eA}	Spoiled[2]	Spoiled
SFLSD	18h at 20°C	0.52±0.01 ^{dC}	0.54±0.01 ^{dC}	0.56±0.01 ^{dB}	0.57±0.01 ^{dB}	0.58±0.01 ^{dA}	Spoiled
SFHSD		0.72±0.01 ^{bD}	0.73±0.01 ^{bC}	0.74±0.01 ^{bC}	0.76±0.01 ^{bB}	0.77±0.00 ^{bAB}	0.78±0.01 ^{bA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-f}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A\ -D}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] Key of the table as Table 6.3

[2] Spoiled: Microbial growth was observed on the surface of the breads

6.3.7 Physical and chemical properties of bread samples

The physical and chemical properties were measured after baking bread on day 0 as shown in Table 6.12. There were significant differences (P<0.05) in the loaf volume among the treatments, except bread with FFLSD and SFHSD, where the values ranged between 881.67 to 1480ml. There were significant differences (P<0.05) in the height of breads among all the treatments, except breads with FFCON and SFHSD; the height of the bread with FFHSD was significantly higher than the other treatments. The moisture percentage of the breads ranged from 36.55-44.64% and water activity from 0.92–0.95. There were no significant differences (P>0.05) in the moisture between breads with SFCON, SFLSD and SFHSD. The bread with FFHSD had a lower moisture percentage and water activity than the other treatments. The results showed that the cell total area percentage of all treatments at 30ºC for 3h of fermentation was higher compared to the treatments at 20ºC for 18h. The cell-total area of the breads with FFLSD and FFHSD was higher than the other treatments. No change showed in the cell-total area between controls (FFCON and SFCON) and between bread with SFCON and SFHSD: Figure 6.4 shows the pores area of the bread samples after conversion to grey and then to black and white.

Table 6.12: physical and chemical properties* of bread with added different concentration and fermentation process of sourdough

Treatment**	Fermentation process	Loaf volume (ml)	Height (mm)	Moisture (%)	Aw	Cell-total area %
FFCON	3h at 30°C	1113.33±5.51 ^{c}	95.33±1.00 ^{c}	40.57±1.71 ^{bc}	0.95±0.002 ^{a}	13.06±0.86 ^{b}
FFLSD		1225.67±6.66 ^{b}	106.00±1.00 ^{b}	39.72±1.14 ^{c}	0.93±0.002 ^{c}	16.67±1.19 ^{a}
FFHSD		1480.00±12.12 ^{a}	115.00±1.00 ^{a}	36.55±1.16 ^{d}	0.92±0.002 ^{d}	17.53±1.31 ^{a}
SFCON	18h at 20°C	881.67±10.60 ^{e}	87.67±0.58 ^{e}	44.64±0.31 ^{a}	0.95±0.001 ^{a}	11.77±0.76 ^{bc}
SFLSD		1019.33±6.11 ^{d}	91.00±1.00 ^{d}	43.33±0.28 ^{ab}	0.94±0.002 ^{b}	7.32±0.86 ^{d}
SFHSD		1211.67±5.69 ^{b}	97.67±0.58 ^{c}	43.30±0.81 ^{ab}	0.94±0.001 ^{b}	10.25±0.62 ^{c}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-e}$ Means in the same column with different superscripts are significantly different (P<0.05).

** Key of the table as Table 6.3

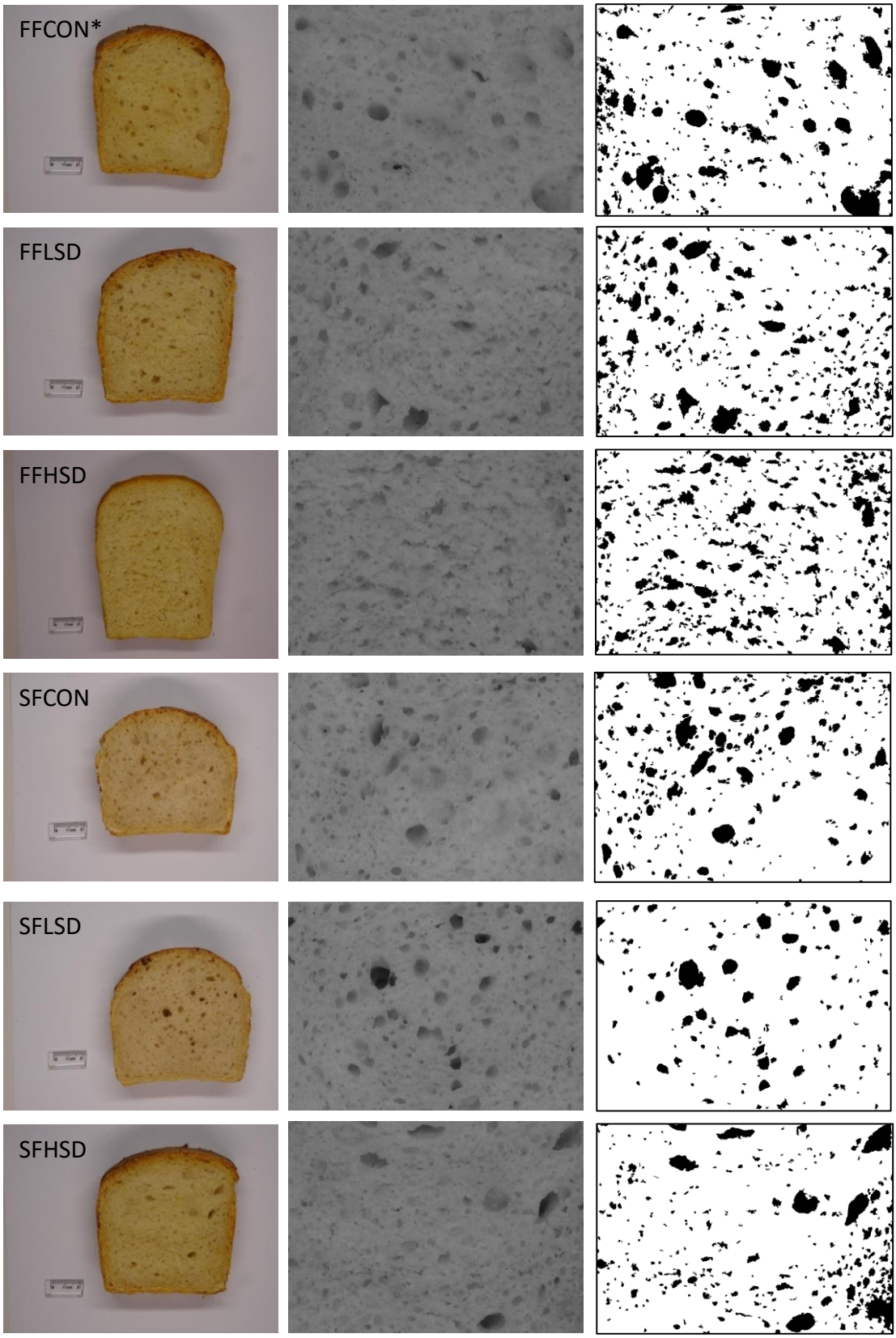


Figure 6.4: The areas of pores of the crumb breads measurement by using ImageJ software. * Key of the figure as Table 6.3

6.3.8 Texture properties of bread samples during storage time

Bread properties were measured over a storage period, including textures such as hardness, springiness and chewiness for sourdough breads as shown in Table 6.13. On day 0, hardness and chewiness recorded the same effect which showed that bread with FFHSD significantly (p<0.05) decreased these factors. In contrast, hardness and chewiness of the bread with SFCON significantly increased comparing to the other treatments. There were no significant differences (P>0.05) in the springiness among all the treatment except SFCON with SFLSD. On all other days, hardness of bread with FFHSD was shown to be lower than the other treatments. However all breads which made with sourdough addition at low and high level and with both fermentation processes were softer than the control breads at each day of storage. On day 2, springiness was significantly higher and chewiness lower in the bread with FFHSD compared to the other treatments. On day 4, bread with FFHSD had higher springiness, while, the chewiness of bread with FFHSD, SFLSD and SFHSD was lower. On days 6 and 8, there were no significant differences in springiness from all breads with added sourdough except bread with FFLSD on day 6. Furthermore, hardness and chewiness were increased in all treatments over 10 days of storage at room temperature. Microbial growth was observed on the surface of bread with SFCON on day 8 and breads with FFCON, SFCON and SFLSD on day 10 of storage. They were discarded and removed from the test.

Table 6.13: The influence of the sourdough on the quality* of breads

Time (day)	Treatment**	Hardness (g)	Springiness	Chewiness
0	FFCON	176.61±4.78 ^{b}	0.95±0.02 ^{ab}	121.46±2.28 ^{c}
	FFLSD	157.65±6.41 ^{c}	0.94±0.02 ^{ab}	100.30±2.81 ^{d}
	FFHSD	113.60±1.42 ^{e}	0.95±0.02 ^{ab}	90.61± 3.72 ^{e}
	SFCON	203.08±3.18 ^{a}	0.93±0.01 ^{b}	144.19±2.50 ^{a}
	SFLSD	174.39±3.82 ^{b}	0.96±0.02 ^{a}	134.41±4.29 ^{b}
	SFHSD	136.10±6.93 ^{d}	0.95±0.01 ^{ab}	122.29±3.54 ^{c}
2	FFCON	282.89±6.98 ^{b}	0.94±0.01 ^{bc}	188.37±4.11 ^{b}
	FFLSD	225.74±5.10 ^{d}	0.95±0.02 ^{b}	161.74±3.42 ^{c}
	FFHSD	192.49±9.13 ^{e}	0.98±0.01 ^{a}	108.79±4.77 ^{e}
	SFCON	307.87±7.11 ^{a}	0.92±0.01 ^{c}	206.12±5.26 ^{a}
	SFLSD	256.94±4.19 ^{c}	0.92±0.01 ^{c}	160.65±6.53 ^{c}
	SFHSD	232.47±5.29 ^{d}	0.93±0.01 ^{c}	150.93±7.01 ^{d}
4	FFCON	376.98±5.09 ^{a}	0.91±0.01 ^{b}	207.30±6.79 ^{b}
	FFLSD	295.50±4.07 ^{d}	0.93±0.01 ^{b}	189.34±8.95 ^{c}
	FFHSD	244.99±5.51 ^{f}	0.95±0.01 ^{a}	167.54±8.41 ^{d}
	SFCON	337.78±6.47 ^{b}	0.88±0.01 ^{c}	224.45±7.53 ^{a}
	SFLSD	314.81±5.32 ^{c}	0.92±0.01 ^{b}	165.18±12.54 ^{d}
	SFHSD	271.93±4.68 ^{e}	0.93±0.01 ^{b}	162.46±7.44 ^{d}
6	FFCON	492.19±6.83 ^{b}	0.88±0.01 ^{a}	242.61±6.77 ^{b}
	FFLSD	365.38±5.24 ^{d}	0.91±0.01 ^{b}	216.13±8.46 ^{c}
	FFHSD	315.84±4.98 ^{f}	0.92±0.01 ^{b}	182.02±9.44 ^{d}
	SFCON	570.08±5.81 ^{a}	0.87±0.01 ^{a}	279.67±7.23 ^{a}
	SFLSD	402.33±5.12 ^{c}	0.91±0.01 ^{b}	238.13±10.66 ^{b}
	SFHSD	340.79±7.00 ^{e}	0.92±0.01 ^{b}	204.37±10.13 ^{c}
8	FFCON	608.61±8.43 ^{a}	0.87±0.01 ^{b}	298.01±7.20 ^{a}
	FFLSD	459.75±6.87 ^{d}	0.88±0.01 ^{b}	264.22±5.99 ^{c}
	FFHSD	389.96±6.69 ^{e}	0.91±0.00 ^{a}	230.86±6.84 ^{d}
	SFLSD	526.12±5.27 ^{b}	0.91±0.01 ^{a}	284.18±7.38 ^{b}
	SFHSD	484.00±9.80 ^{c}	0.92±0.01 ^{a}	238.04±6.29 ^{d}
10	FFLSD	511.80±5.05 ^{b}	0.86±0.02 ^{b}	286.47±6.61 ^{b}
	FFHSD	441.90±6.85 ^{c}	0.90±0.00 ^{a}	263.29±7.87 ^{c}
	SFHSD	569.79±5.37 ^{a}	0.86±0.01 ^{b}	296.92±8.58 ^{a}

* $^{a-f}$ Means (n=6) in the same column with different letters in each separate days are significantly different ($P < 0.05$). ** Key of the table as Table 6.3

6.3.9 Colour of bread samples

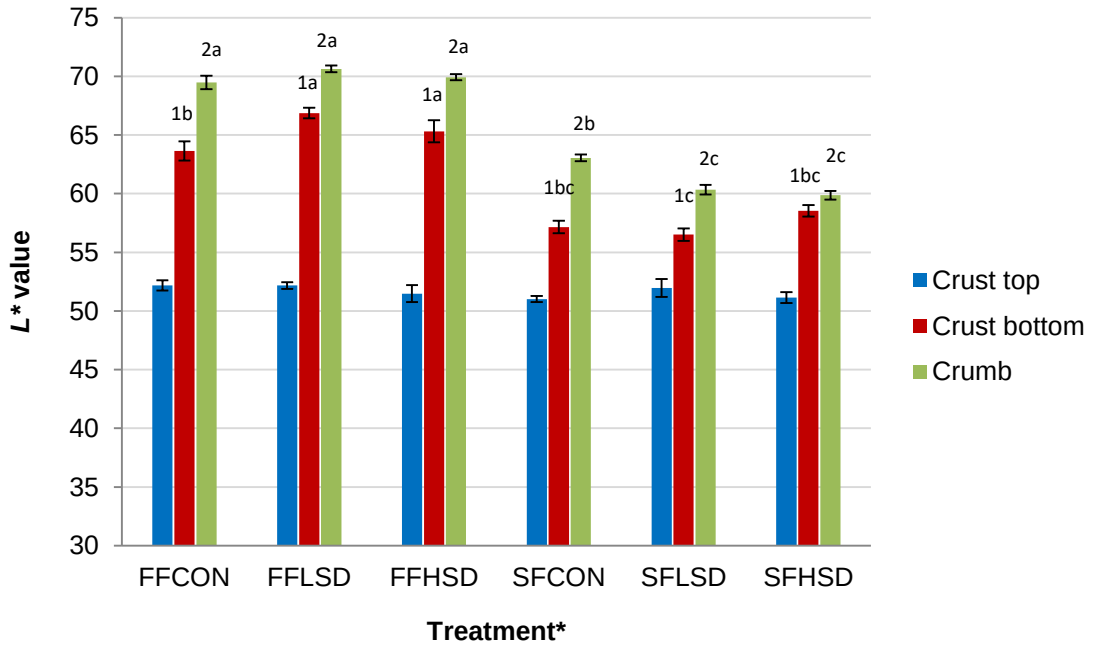
Whiteness of crust (top and bottom) and crumb of breads are presented in Table 6.14 and L^* (lightness) a^* (redness) and b^* (yellowness) measurements of crust (top and bottom) and crumb of breads are showed in Figure 6.5. Bread with FFHSD had a significantly lower (P<0.05) whiteness of crust top. There were no significant differences of whiteness of crust bottom between breads with SFCON and SFLSD, and no differences between FFCON and FFLSD, and also FFCON, FFHSD and SFHSD. No changes on the whiteness of crumb colour were noticed in the fast fermented breads with and without sourdough addition. The crumb colour of breads with SFLSD and SFHSD had noticeably lower whiteness and lightness than the other treatments. No change was found in lightness on the crust top of breads among all the treatments. Redness of the crust top of bread with FFCON and FFHSD was higher than the other treatments, while the crumb of bread with SFHSD had noticeably higher redness. There was significantly decreased yellowness of the crust top of the bread with SFLSD and SFHSD. There were no changes in yellowness of the crust bottom of breads with SFCON, SFLSD and FFHSD, and lower yellowness of crumb of bread with FFCON was found.

Table 6.14: Whiteness crust and crumb colour* of sourdough bread

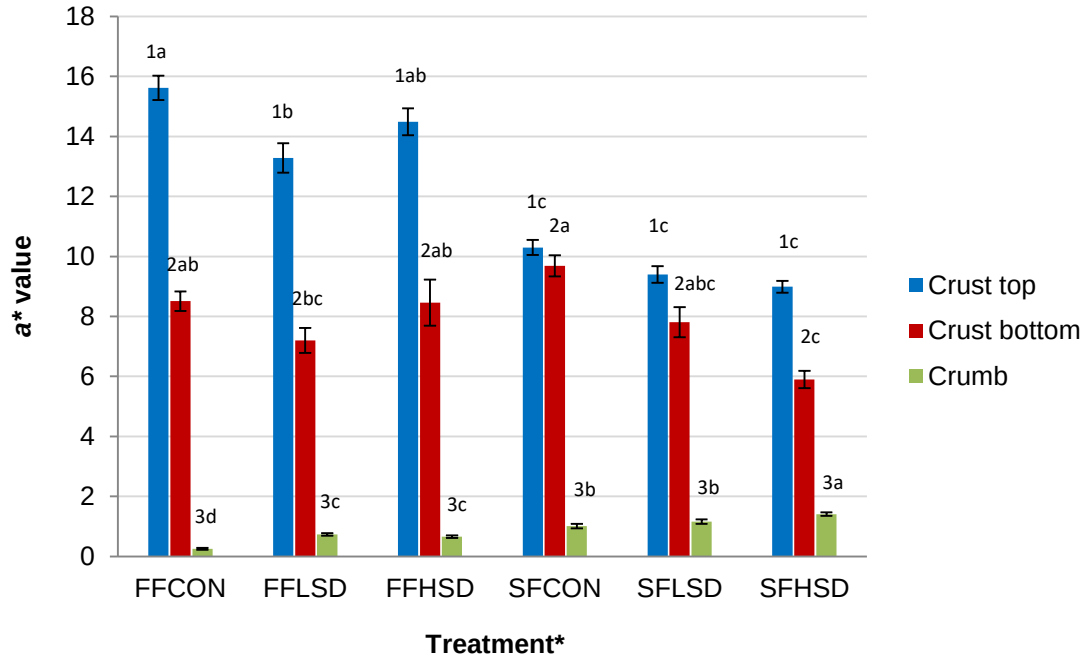
Treatment**	Fermentation process	Crust colour		Crumb colour
		Top	Bottom	
FFCON	3h at 30°C	41.00±2.00 ^{b}	-48.20±1.28 ^{ab}	66.27±1.54 ^{a}
FFLSD		44.24±1.35 ^{a}	-45.63±1.65 ^{a}	66.37±0.75 ^{a}
FFHSD		38.71±1.74 ^{c}	-49.14±4.28 ^{b}	65.46±0.76 ^{a}
SFCON	18h at 20°C	42.18±0.92 ^{ab}	-56.06±1.88 ^{c}	59.75±0.91 ^{b}
SFLSD		43.29±1.95 ^{a}	-56.16±1.96 ^{c}	56.86±1.17 ^{c}
SFHSD		42.54±0.93 ^{ab}	-51.34±1.54 ^{b}	57.06±1.02 ^{c}

* Mean values from nine replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a\text{-}c}$ Means within a column with different superscripts differ significantly (P<0.05).
** Key of the table as Table 6.3

(a) Lightness



(b) Redness



(c) Yellowness

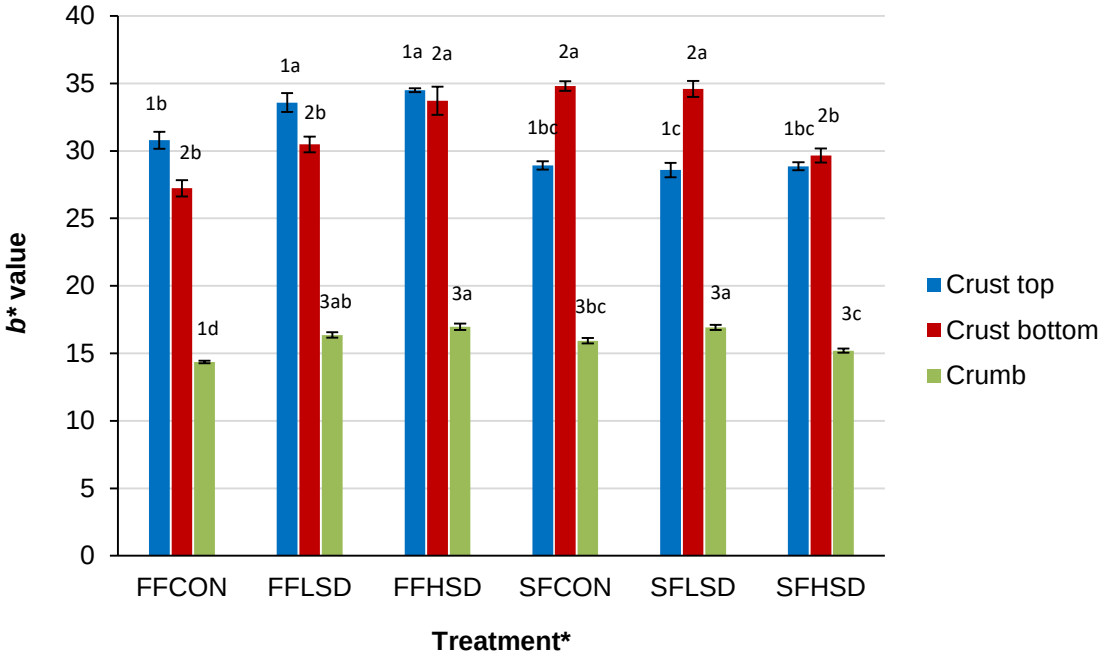


Figure 6.5: Colour (a) lightness, (b) redness and (c) yellowness values for crust (top and bottom) and crumb of bread samples. Mean values from nine replicates ± standard deviations (ANOVA was followed by Turkey's test). [a-c] Means within same parameter with different superscripts are significantly different (P<0.05). * Key of the treatments as Table 6.3

6.3.10 Shelf life of bread samples

Shelf life of the sourdough breads was determined by microbial growth over 10 days of storage at room temperature. The aerobic plate count (APC) and LAB were observed in all bread samples as shown in Table 6.15 and 6.16 respectively. On day 0, the growth of APC ranged from 3.52 to 4.65 $\log_{10}$CFU/g and LAB from 3.49 to 3.98 $\log_{10}$CFU/g while the number of APC in bread with SFCON significantly was higher (P<0.05) and the number of LAB was lower. There were significant differences between the breads at each day of storage except growth of APC in the bread FFLSD with SFHSD on days 2, 4 and 6 and also growth of LAB in the breads with SFLSD with SFHSD on day 0. The growth of APC and LAB were significantly increased in all the treatments over 10 days of storage at room temperature. The bread with SFCON on days 8 and 10, and breads with FFCON and SFLSD on day 10 of storage were discarded and removed from the test due to the microbial growth on the surface of them.

Table 6.17 shows the growth of mould and yeast over 10 days of storage at room temperature. On day 0, there was no growth of mould and yeast detected in any of the treatments. No mould and yeast colonies were detected when the breads with FFLSD, FFHSD and SFHSD were tested on day 2 of storage at room temperature. The number of mould and yeast in the bread with SFCON was significantly higher in comparison with the other treatments over days of storage at room temperature until spoiled by microbial growth on the surface of the bread.

Table 6.15: Aerobic plate count (APC) of sourdough bread over 10 days of storage at room temperature (*Log_{10} CFU/g)

Treatment[1]	Fermentation process	Time (days)					
		0	2	4	6	8	10
FFCON	3h at 30°C	4.65±0.03 ^{bE}	5.01±0.01 ^{bD}	5.30±0.02 ^{bC}	5.65±0.02 ^{bB}	6.17±0.02 ^{aA}	Spoiled
FFLSD		3.84±0.01 ^{dF}	4.11±0.02 ^{dE}	4.31±0.02 ^{dD}	4.64±0.02 ^{dC}	5.12±0.01 ^{dB}	5.77±0.01 ^{bA}
FFHSD		3.52±0.02 ^{fF}	3.92±0.02 ^{eE}	4.20±0.02 ^{eD}	4.46±0.01 ^{eC}	4.91±0.02 ^{eB}	5.21±0.02 ^{cA}
SFCON	18h at 20°C	4.94±0.01 ^{aD}	5.13±0.02 ^{aC}	5.42±0.01 ^{aB}	6.03±0.02 ^{aA}	Spoiled[2]	Spoiled
SFLSD		3.95±0.02 ^{cE}	4.22±0.02 ^{cD}	4.45±0.02 ^{cC}	4.82±0.02 ^{cB}	5.56±0.02 ^{bA}	Spoiled
SFHSD		3.57±0.02 ^{eF}	4.07±0.02 ^{dE}	4.29±0.02 ^{dD}	4.63±0.03 ^{dC}	5.25±0.02 ^{cB}	6.08±0.02 ^{aA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-f}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-F}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] Key of the table as Table 6.3

[2] Spoiled: Microbial growth was observed on the surface of the breads

Table 6.16: Growth of LAB of sourdough bread over 10 days of storage at room temperature (*Log_{10}CFU/g)

Treatment[1]	Fermentation process	Time (days)					
		0	2	4	6	8	10
FFCON	3h at 30°C	3.57±0.01 ^{dE}	3.94±0.01 ^{eD}	4.09±0.02 ^{eC}	4.30±0.01 ^{eB}	4.82±0.02 ^{eA}	Spoiled
FFLSD		3.68±0.02 ^{bF}	4.16±0.01 ^{bE}	4.41±0.01 ^{bD}	4.90±0.02 ^{bC}	5.10±0.01 ^{bB}	5.40±0.02 ^{bA}
FFHSD		3.98±0.01 ^{aF}	4.32±0.01 ^{aE}	4.94±0.01 ^{aD}	5.23±0.01 ^{aC}	5.44±0.01 ^{aB}	5.90±0.02 ^{aA}
SFCON	18h at 20°C	3.49±0.01 ^{eD}	3.82±0.02 ^{fC}	4.04±0.02 ^{fB}	4.26±0.01 ^{fA}	Spoiled[2]	Spoiled
SFLSD		3.60±0.01 ^{cE}	3.98±0.01 ^{dD}	4.24±0.02 ^{dC}	4.57±0.01 ^{dB}	4.90±0.02 ^{dA}	Spoiled
SFHSD		3.62±0.01 ^{cF}	4.02±0.01 ^{cE}	4.36±0.01 ^{cD}	4.64±0.02 ^{cC}	5.04±0.01 ^{cB}	5.32±0.02 ^{cA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-f}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-F}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] Key of the table as Table 6.3

[2] Spoiled: Microbial growth was observed on the surface of the breads

Table 6.17: Mould and yeast counts of sourdough bread over 10 days of storage at room temperature (*Log_{10}CFU/g)

Treatment[1]	Fermentation process	Time (days)					
		0	2	4	6	8	10
FFCON	3h at 30°C	n.d.[2]	2.60±0.02 ^{bD}	3.13±0.01 ^{bC}	3.88±0.02 ^{bB}	4.57±0.02 ^{aA}	Spoiled
FFLSD		n.d.	n.d.	2.64±0.02 ^{dD}	3.03±0.02 ^{eC}	3.74±0.02 ^{dB}	4.29±0.01 ^{bA}
FFHSD		n.d.	n.d.	2.50±0.01 ^{eD}	2.90±0.01 ^{fC}	3.24±0.02 ^{eB}	4.03±0.02 ^{cA}
SFCON	18h at 20°C	n.d.	2.67±0.02 ^{aC}	3.24±0.02 ^{aB}	4.19±0.02 ^{aA}	Spoiled[3]	Spoiled
SFLSD		n.d.	2.50±0.02 ^{cD}	3.09±0.02 ^{cC}	3.46±0.01 ^{cB}	4.10±0.02 ^{bA}	Spoiled
SFHSD		n.d.	n.d.	2.64±0.02 ^{dD}	3.26±0.02 ^{dC}	3.85±0.02 ^{cB}	4.42±0.02 ^{aA}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-f}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-D}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] Key of the table as Table 6.3

[2] n.d.: not detected, the detection limit was <10

[3] Spoiled: Microbial growth was observed on the surface of the breads

The growth of *Bacillus cereus* in bread samples during storage periods at room temperature is shown in Table 6.18. *Bacillus cereus* was not detected in breads which were fermented with sourdoughs including FFLSD, FFHSD, SFLSD and SFHSD until day 8 of the storage period, except bread with SFLSD where they were found growing on day 8 of the storage period at room temperature. They were found on control breads including FFCON and SFCON on day 6 of the storage at room temperature. On day 10, the growth of *Bacillus cereus* in the bread with FFHSD was significantly lower ($P<0.05$) compared to the other treatments. *Bacillus cereus* was increased significantly in all treatments during storage periods at room temperature.

The bread samples were tested for spore forming bacteria, which were not detected in any bread samples in any case of storage time, which might be due to having been baked at 220°C for 20min. Spores of *Bacillus* species observed in flour and other raw components are resistant to temperature and some of them can survive the baking process where the temperature in the centre of the crumb reaches up to 97–101°C for a few minutes (Valerio *et al*., 2012; Rosell *et al*., 2016). During baking process, most microbes are killed

Bread with SFCON and FFCON, which were made without sourdough addition (control bread), had a shelf life of 6 and 8 days respectively; this change might be due to the slow and fast fermentation at different temperature. However, the bread with fast fermentation at level 9 and 18% and slow fermentation at level 18% of sourdough addition had a shelf life of 10 days when tested for the level of APC, LAB, mould and yeast colony counts, growth of *Bacillus cereus* and spore forming bacteria. The reason is probably due to metabolites of LAB during the fermentation of sourdough and addition to the bread formulation

which have antimicrobial activity against food pathogenic and spoilage microorganisms.

Table 6.18: Growth of *Bacillus cereus* of sourdough bread over 10 days of storage at room temperature (*Log_{10}CFU/g)

Treatment[1]	Fermentation process	Time (days)		
		6	8	10
FFCON	3h at 30°C	2.70±0.02 ^{bB}	3.16±0.02 ^{aA}	Spoiled
FFLSD		n.d.[2]	n.d.	2.87±0.01 ^{a}
FFHSD		n.d.	n.d.	2.6±0.01 ^{c}
SFCON	18h at 20°C	2.80±0.01 ^{a}	Spoiled[3]	Spoiled
SFLSD		n.d.	2.95±0.01 b	Spoiled
SFHSD		n.d.	n.d.	2.81±0.02 ^{b}

* Mean values from three replicates ± standard deviations (ANOVA was followed by Turkey's test). $^{a-c}$ Means in the same column with different superscripts are significantly different (P<0.05). $^{A-B}$ Means in the same row with different superscripts are significantly different (P<0.05).

[1] Key of the table as Table 6.3

[2] n.d.: not detected, the detection limit was <10

[3] Spoiled: Microbial growth was observed on the surface of the breads

6.3.11 Sensory evaluation of bread samples

The current sensory evaluation was undertaken to assess overall appearance, aroma, texture, flavour, acidity and overall acceptability of the breads with FFCON, FFLSD, FFHSD, SFCON, SFLSD and SFHSD as the labels show in Figure 6.6. In a ranking test, the higher scores refer to a food with high acceptability.

Of the six sensory attributes evaluated, only one attribute (acidity) showed no significant change (P>0.05) between all the treatments. On the other hand, there is a considerable difference between other attributes. For overall appearance and overall acceptability, bread with FFLSD recorded the highest average rank, scoring 122.8 and 128 respectively. Those were significantly different than the breads with SFLSD and SFHSD. There were also significant differences between FFLSD with SFCON in the term of overall acceptability, but those were not significantly different to the other breads. For texture, bread with SFHSD recorded the lowest average rank with 66.7 and it was significantly different compared to the other breads. In both aroma and flavour, the breads with fast fermentation had a higher average rank than breads with slow fermentation. However, the statistical analysis indicated that there was no significant difference in aroma between the breads with FFHSD, SFCON, and SFLSD and also between FFCON, SFCON, and SFLSD in flavour. The results are shown in Figure 6.6 and pairwise comparisons for the sensory attributes were used for comparison between treatments which is shown in Figure 6.7.

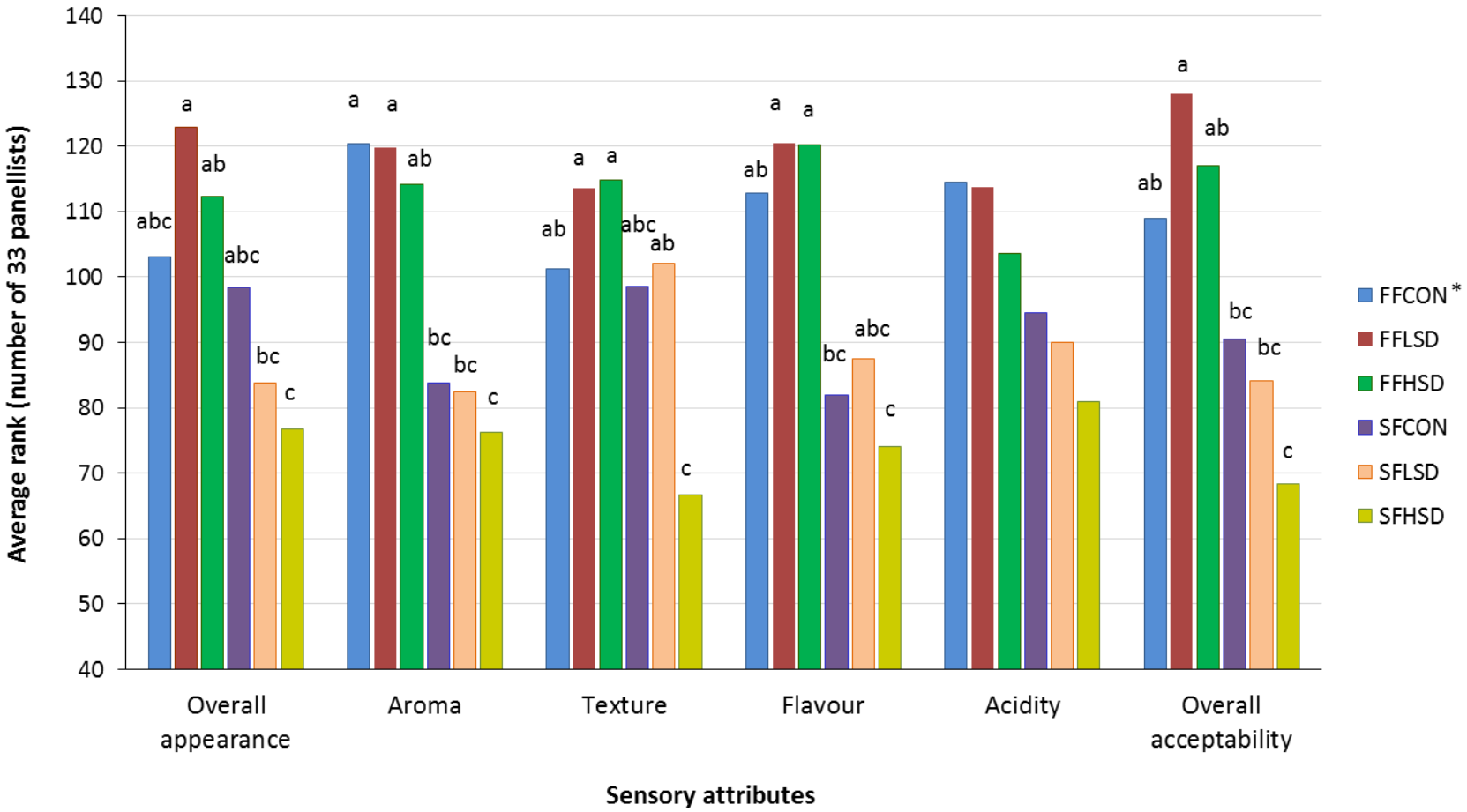


Figure 6.6: Average rank of sensory evaluation of breads made with and without sourdough using fast fermented process at 30ºC for 3h and slow fermented process at 20ºC fore 18h with different concentration of sourdough

* Key of the figure as Table 6.3

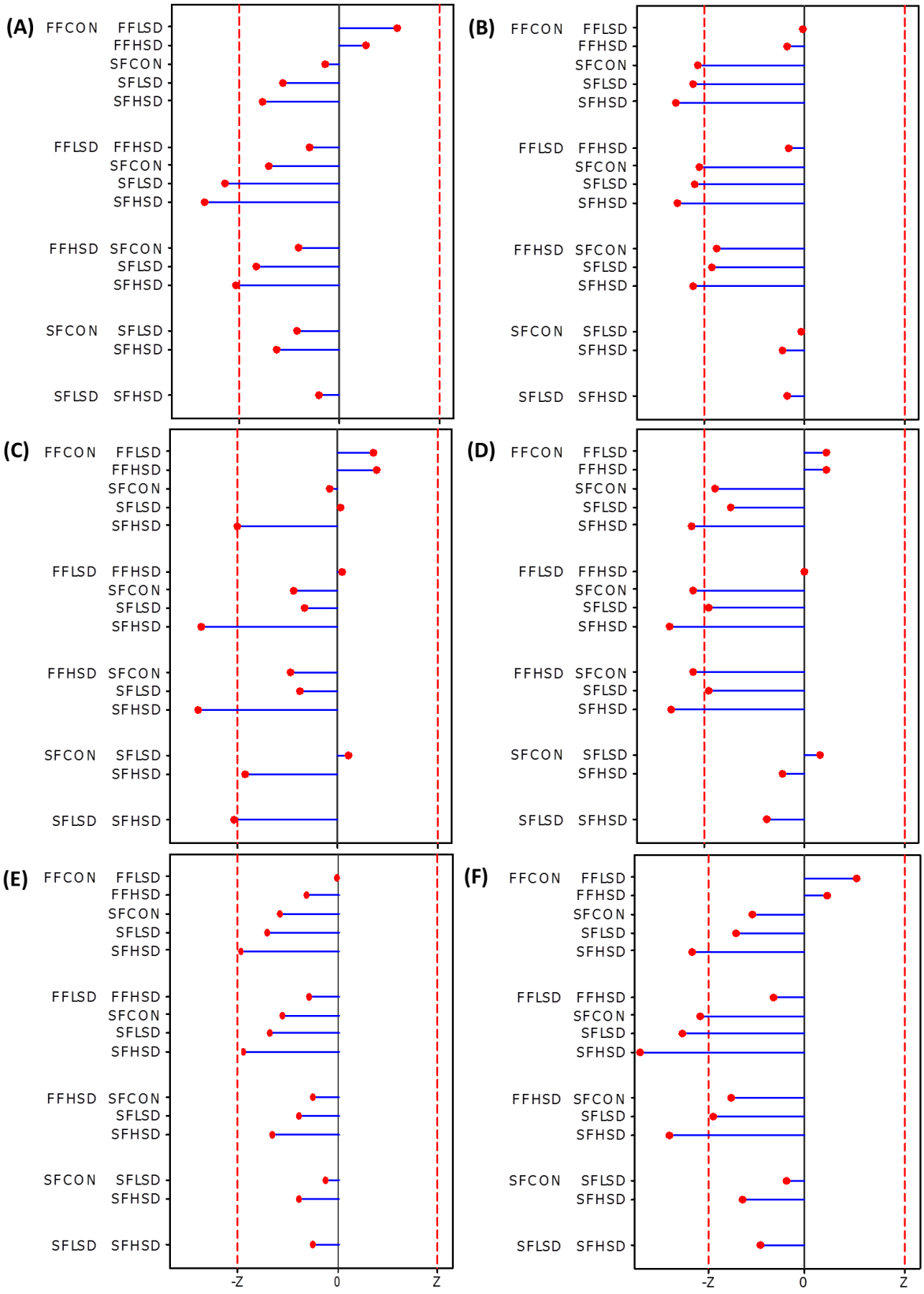


Figure 6.7: Pairwise comparisons (normal (0,1) distribution) of the sensory attributes of breads using Donn's test (|Bonferroni Z-value| : 2.475). (A) Overall appearance, (B) Aroma, (C) Texture, (D) Flavour, (E) Acidity, (F) Overall acceptability. The y-axis labels are listed on Table 6.3.

6.4 Discussion

Starters *Lb*. *brevis*, *Lb*. *rossiae* and *Lb*. *plantarum* as a single and mixed culture and also 17 sourdough samples separately were tested for sourdough fermentation to evaluate sourdough bread making properties.

The pH and TA values of the sourdoughs were tested before and after day five of back-slopping at 30ºC for 24h. The pH remain constant, showing no changes before starting the fermentation. During the fermentation time at 30ºC for 24h, pH of sourdoughs decreased and acidity increased due to the presence of LAB. This result is in agreement with the results of Banu *et al*. (2010) who reported that fermented sourdough with LAB effected to decrease pH and increase acidity and also cause differences in the pH and acidity between fermented sourdoughs, which might be due to use of different starter culture and flour spontaneous microflora. These results in the present study are in agreement with the findings of Thiele *et al*. (2002) and Wehrle and Arendt (1998) who studied the wheat sourdough and found that the pH ranged from 3.5-4.3 as observed in the present study. However, Robert *et al*. (2006) reported that the pH of sourdoughs change during sourdough fermentation when using different LAB starters, while, Jekle *et al*. (2010) showed small variations in the pH and acidity with the use of different starter cultures during sourdough fermentation.

Growth of LAB, APC and yeasts (each separately when tested) were different between sourdough samples; the number of LAB in sourdoughs prepared with starter cultures was higher than the other microorganisms. The results agree with Paramithiotis *et al*. (2005) who reported that the level of starter cultures was higher than yeast during sourdough fermentation. In addition, the results

agree with the findings of Meignen *et al*. (2001) and Stolz (2003) who reported that the level of LAB in sourdough is between 10^8-10^9 CFU/g, and the LAB: yeast ratio is generally 100:1. In the present study, the level of LAB in the fermented control dough was about 10^4-10^5 CFU/g, which is in the range found in fermented dough without the addition of starter cutlures as presented by Palacios *et al*. (2006). The high number of LAB in prepared sourdoughs might be due to the addition of LAB as starter cultures to prevent growth of other microbes at fermentation time.

The results of the present study show that LAB strains, when used for sourdough fermentation, were capable of producing organic acids. Different quantities of organic acids (lactic acid, acetic acid and propionic acid) are produced by LAB and this could be due to the utilization of both homo and heterofermentative starter cultures of LAB for making sourdough samples. Production of suitable metabolites during sourdough fermentation depends on the availability of soluble carbohydrates in the flour (Collar, 1996; Martínez-Anaya, 1996). In some sourdough samples, the results were in agreement with Sterr *et al*. (2009) and Minervini *et al*. (2012) who reported that the lactic acid production in the fermented sourdoughs is the dominant product which ranged from 63.7 to 178.1 mm/L, and acetic acid and propionic acid are produced as well. Arendt *et al*. (2007) showed that organic acids affect the protein and starch fractions of the flour. In addition, the drop in pH associated with acid production in fermented sourdough causes an increase in the amylase and protease enzyme activities of the flour, thus leading to a reduction in staling of bread products (Arendt *et al*., 2007).

In the present study, PCR-DGGE was applied to identify microorganisms in

fermented sourdough samples, which is an easy and reliable technique that gives easy-to-read results. The phylogenetic situation of LAB as it is currently presented is based on 16S rRNA genes and shows a clear separation of the genera. The DGGE gel band numbers in the dough control was higher than the sourdough treatments. The low DGGE gel band numbers in the sourdough treatments might be due to the addition of LAB as starter cultures to prevent growth of other microbes at fermentation time. Also, refreshment (back-slopping) of the sourdough could have a possible impact on the microbial communities via changes in metabolic activity of the microbial communities at all times (De Vuyst and Vancanneyt, 2007). However, the environment could be affected in the dough by microorganisms which may come from air, dust, water, handling and processing equipment. Some researchers showed that the microorganisms usually originate from flour, dough ingredients or the environment (De Vuyst and Neysens, 2005; Gobbetti *et al*., 2005; Paramithiotis *et al*., 2006; Corsetti and Settanni, 2007). The present study shows that the *Lactobacillus* species were the dominant group in wheat sourdough as the results are in agreement with the findings of Ricciardi *et al*. (2005) and Corsetti *et al*. (2001). Band number 13 was identified as *Clostridium saccharobutylicum* strain NCP 262. The presence of this species can be explained by the absence of LAB; it could also be argued that *Clostridium saccharobutylicum* can be found as a result of anaerobic conditions.

The use of sourdough to make breads is to improve the bread properties, and to increase the safety and the shelf life of breads through the reduction in growth of pathogenic microorganisms and bacterial sporulation. The breads were prepared with different concentrations of sourdough and different temperatures

and fermentation times. Some researchers have reported that the process parameters such as dough yield, number of back slopping of the sourdough, temperature and fermentation time of the dough affect the quality and shelf life of the bread products (Hammes and Gänzle, 1998; Meroth *et al*., 2003b; Vogelmann and Hertel, 2011; Vrancken *et al*., 2011). The pH of the dough fermented with sourdough decreased and the acidity increased during fermentation at different temperatures (3h at 30ºC and 18h at 20ºC). The results also show that the pH of each treatment decreased and the TA of each treatment increased at 30ºC for 3h of fermentation as compared to the same treatment at 20ºC for 18h.

The changes from carbohydrates to organic acids can be explained by presence of LAB, which subsequently can lead to a decrease in pH and increase the acidity values. Some researchers also found that the sourdough addition to the dough could cause a pH decrease and subsequent increase in acidity (Gül *et al*., 2005; Katina *et al*., 2009). Under most circumstances, the LAB play an important role in dough and bread acidity (Hutkins, 2006). During the storage period at room temperature, there were significant decreases in the pH and increases in the acidity values for all treatments. The change during recent periods of storage may be caused by increased growth of microorganisms on the bread samples which may have decreased the pH value in the food products.

The results show that the loaf volume and height of bread with FFHSD was significantly higher than the other treatments, which due to use of high concentration of sourdough and possibly the use of 30ºC for proofing the dough was better suited to growth of LAB and production of metabolites. This result is

in agreement with the findings of Corsetti *et al*. (2000) who showed the effectiveness of LAB as starters for fermentation of dough in improving bread volume when compared to the fermented dough with pure yeast, which might be due to interactions of metabolic compounds produced by LAB and yeasts during fermentation of the dough. Also, Clarke *et al*., (2002) reported that sourdough addition in bread production effects an increase in loaf specific volume and has a positive effect on the crumb structure. The loaf volume and height of the breads with FFLSD and FFHSD were significantly higher than the breads with SFLSD and SFHSD. The slow fermentation at 20ºC for 18h had low acidification as improved volume of the sourdough breads has been suggested to be dependent on the nature and intensity of the acidification process (Clarke *et al*., 2003). The loaf volume improvement in breads with FFLSD and FFHSD might be due to the ability of heterofermentative LAB to produce more CO_2 in the acidified dough as reported by Gobbetti *et al*. (1995b) and Katina *et al*. (2006a).

In the present study, there were no significant differences of the moisture and water activity of breads with slow fermented and low temperature among treatments, except the SFCON bread which was higher than the breads with SFLSD and SFHSD, while the moisture and water activity of bread with FFHSD was significantly lower than the other treatments. Barber *et al*. (1992) reported that strains of LAB had no affect on the variation of moisture during baking and storage. The change of moisture and water activity might be due to the LAB activities, fermentation time and using different temperatures for microbial growth. The fermented sourdough influences moisture redistribution throughout the loaf during storage as reported by Corsetti *et al*. (2001). Water activity has a

marked effect on the growth of microorganisms. The breads with FFLSD and FFHSD had a higher ratio of pore area comparing to the other treatments. It could be the result from interactions of metabolites which were produced by LAB and yeasts during fermentation of the sourdoughs. The microorganisms present during cereal fermentation can produce metabolites, which consequently interact with the grain constituents. For example, LAB produce lactic and acetic acids, and yeasts produce carbon dioxide and ethanol; interaction between them can be important for the metabolic activity of the sourdough (Poutanen *et al*., 2009). The result is in agreement with the findings of Coda *et al*. (2010) who demonstrated that the highest cell total area of crumb bread was found in bread with added sourdough. The lowest hardness and chewiness of crumb slices were found in breads with FFHSD. Sourdough breads which were made with both fermentation processes were softer than the control breads on all days of storage. However, it was pointed out that the bread made with sourdough starters was softer when compared to the other breads as reported by Dal Bello *et al*. (2007) and Coda *et al*. (2010). It could be noticed quite clearly that the addition of sourdough at different concentrations with slow (20ºC for 18h) and fast (30ºC for 3h) fermentation time could reduce hardness and chewiness in general to a good extent. Hardness and chewiness of breads increased and springiness decreased over storage time.

This study shows that the whiteness of the crust top of the bread with FFLSD was lower than the other treatments. No changes were found in whiteness crumb colour between breads which were fast fermented at 30ºC for 3h (FFCON, FFLSD and FFHSD), whereas, crumb breads with SFLSD and SFHSD had significantly lower whiteness. Chiavaro *et al*. (2008) reported that

the bread with sourdough had lower lightness values for crust colour with no significant differences in the crumb colour. Changes in the crust and crumb colour of the bread samples may be related to the production of different compounds during the fermentation process by microorganisms. However, bread colour and aroma develop during baking, simultaneously with crust formation, and derive from chemical reactions such as maillard and sugar caramelisation (Ahrné *et al*., 2007).

While investigating the shelf life of bread, microbial growth bread was tested over a 10 days storage period at room temperature. The breads with FFLSD, FFHSD and SFHSD were unspoil after 10 days, whereas the breads with FFCON and SFLSD had a shelf life of 8 days, and SFCON had 6 days shelf life. The present study is in agreement with Saikia and Sit (2014) who reported that the sourdough bread had a shelf life of 8-9 days compared to a control bread with microbiological shelf life of 5 days. The reason for this increase in the shelf life of sourdough bread seems to be linked to antimicrobial components produced by LAB during fermentation which reduce the growth of mould and bacteria (Dal Bello *et al*., 2007). The microbial growth or breadincreased during the storage at room temperature in all cases.. Natural sourdoughs are commonly used in bread-making processes, especially for organic bread. The stability of the sourdough microbial community during and between bread-making processes is debated. However, it has an effective role in bread flavour and dough rise (Lhomme *et al*., 2016). In the present study, sourdough addition for bread production in any case had a lower APC and Mould count compared to the control breads. The growth of mould was not noticed in any treatment on the day of baking. Mould started growing from the bread with FFCON, SFCON

and SFLSD on day 2 of the storage period at room temperature. Similar results found in the results of Moore *et al*. (2008) who reported that the sourdough addition led to significant decrease in mould presence, probably due to antifungal components produced by LAB during fermentation. Sourdough associated LAB produce many antimicrobial substances which reduce the growth of pathogenic microorganisms, such as organic acids, diacetyl, hydrogen peroxide, ethanol, CO_2, fatty acids, phenyllactic acid, reuterin, and fungicins (Messens and De Vuyst, 2002; Schnürer and Magnusson, 2005). Some researchers showed that sourdough *Lb. plantarum* shows very broad antimicrobial activity, and it can produce antifungal compounds such as 4-hydroxyphenlyllactic and phenyllactic acids, which have been identified as responsible for fungal inhibition (Lavermicocca *et al*., 2000; Dal Bello *et al*., 2007; Ryan *et al*., 2008).

The present results show that the sourdough addition has positive effects on the shelf life of bread due to restriction of the growth of *Bacillus cereus*, possibly due to the high acidity value at pH 5 of the sourdough bread from the day of baking over 10 days. The results are in agreement with the results of Katina *et al*. (2002) who clearly demonstrated that the reduction of *Bacillus* counts in the sourdough breads was strongly dependent on the acidity level and particularly at pH 5 of the sourdough that was fermented with *Lb. plantarum*, *P. pentosaceus* or *Lb. brevis*. The toxin of *Bacillus cereus* is restricted in the presence of 0.1% organic acid at pH 5. If the pH outside the cell is less than the pH inside the cell, acid anion will be accumulated within the cell and inhibit cellular function. This is the case in mildly acidic foods (Carpenter and Broadbent, 2009).

Sensory evaluation is an important step in product development to evaluate the success and acceptability of any product by consumers and to predict consumers' perception, which could have an impact on marketability of the products (Elia, 2011). Bread flavour is known to be a key factor for consumers' acceptance and product identification (Rehman *et al*., 2006). This study showed that the results of bread flavour with sourdough addition FFLSD and FFHSD were higher than the bread control (SFCON). This might be due to flavour components which produced by LAB (Chavan and Chavan, 2011). The use of sourdough in wheat bread production clearly improves the dough properties, bread texture and flavour (Hammes and Gänzle, 1998; Martínez-Anaya, 2003). The present study showed that all sensory attributes of breads with slow fermentation at 20ºC for 18h were lower than breads with fast fermentation at 30ºC for 3h, which might be due to the differences in the temperature and fermentation time and their effect on metabolic activity of microbes (Meroth *et al*., 2003b; Vogelmann and Hertel, 2011). No difference was found in the acidity when tasted by panellists. The overall appearance and overall acceptability of breads with FFLSD and FFHSD were higher than the others, which might be due to improve the sensory characteristics such as loaf volume, texture and flavour of breads as described by Rehman *et al*. (2006) and Nawaz *et al*. (2007).

The earliest production of fermented foods was based on spontaneous fermentation resulting from microflora development from that naturally present in the raw material. The quality of the end-product was dependent on the microbial load and the raw material. Moreover, the addition of sourdough with selected starter cultures can cause an improvement in texture properties and a delay in staling of the bread. Antimicrobial compounds from sourdough LAB

have been reported to contribute to extended shelf life of bread products by inhibiting the growth of food spoilage and pathogenic microorganisms. Therefore, any applications involving the sourdough bread as described above will require further investigations to ascertain the most advantageous setting for future use.

6.5 Conclusion

Sourdough fermentation with single and mixed starter cultures was studied to evaluate the quality of a sourdough product. Then, bread with sourdough addition, prepared at different fermentation time and temperature was evaluated for safety and shelf life, quality changes, processability and sensory properties.

Back-slopping fermentations with *Lb. plantarum* JCM1149 (SIN3) had a lower pH and higher acidity values after five days. The high acidification by SIN3, classed as a high acid producer was investigated using HPLC. Even when a single culture was used for sourdough fermentation (SIN3), higher microbial viability was important. PCR-DGGE DNA fingerprinting revealed that in the sourdough samples where starters were used, more diversity was recorded when compared to the control dough. Considering the properties of the sourdoughs, SIN3 was selected for further application studies on bread, where fermentation factors of temperature and rate were considered, in combination with level of sourdough addition. The bread from fast fermentation and high sourdough concentration (FFHSD) had a lower pH, higher acidity and increased quality attributes with significantly better shelf life compared to the other treatments in all cases of storage period (10 days shelf life). Texture

improvement is especially important in production of bread, where the hardness and chewiness of the bread with FFHSD was lower than the other treatments and also exhibited delayed staling during storage. The results of the sensory evaluation revealed that breads with FFLSD and FFHSD showed better acceptability by testing panels for most of the sensory attributes such as overall appearance, texture, flavour and overall acceptability, which might be due to the improvement the bread quality by the addition of sourdough and fermentation processes.

The findings from this study revealed that making bread with an 18% sourdough addition, which was fermented by *Lb. plantarum* (SIN3) as a leavening agent and at a fast fermentation process at 30ºC for 3h of the dough, was successfully affected to produce a bread with good qualities including improved texture, sensory acceptability, loaf volume, height of the bread and decreased moisture and Aw. However, it was effective in increasing the safety and shelf life of the bread product over 10 days by reducing the growth of pathogenic and spoilage microorganisms as well as delaying staling during storage. The developed sourdoughs would allow industries to produce a safe bread with a good quality and increased shelf life without using chemical preservatives.

CHAPTER SEVEN

General discussion, conclusion and future work

7.1 General discussion and conclusion

The preservation of foods by fermentation is a widely practiced traditional technology. Fermentation can help to increase the shelf life and microbial safety of foods. However, the processes by which this takes place are complex and the nature of the bacterial interactions in products are not always well known. The use of LAB as a source of alternative preservatives for food products (e.g. dairy products, bread, fresh fruits, vegetables) have been demonstrated by several studies (Pawlowska *et al*., 2012; Corsetti *et al*., 2015). This has been primarily aimed reducing the growth of food pathogenic and spoilage microorganisms. Consumers have become more demanding about the quality of food. An increasing trend for natural foods, with the minimum amount of chemical additives, is prompting those in the industry to find alternatives (Divya *et al*., 2012). Several natural compounds can inhibit microbial growth and manufacturers can use a limited selection of these additives.

LAB have been associated with the human environment and led to beneficial interactions in food and in the human gut. LAB and their metabolites occur naturally in foods, and sometimes they are used for targeted purposes as preservatives. Moreover, LAB improve the quality of the food products including texture, colour, taste and smell. They could also provide health benefits and reduce economic losses due to spoilage as reported by several previous studies (Settanni and Moschetti, 2010; Gobbetti *et al*., 2014).

The purpose of this work was to investigate the influence of LAB in fermented buttermilk (BM) and sourdough fermentation separately on baked goods. Furthermore, variables in the fermentation process and formulation were evaluated in relation to microbial shelf life, bread texture, colour changes and sensory attributes of bread products.

The first experiment (Chapter 3) demonstrated the antimicrobial effects of four types of BM products fermented with *Lc. lactis* and using commercial nisin (3, 6, 9, 12µg/ml) as reference, against a selection of food pathogenic bacteria including *B. cereus, P. aeruginosa, E. coli* and *S. aureus*. Data in Chapter 3 showed that all BM fermented with *Lc. lactis* indicated antimicrobial effects against pathogenic and spoilage bacteria with different efficacy; Gram-positive bacteria were more sensitive to these, compared to Gram-negative strains. It was explained earlier (Section 1.8.5) that nisin is less effective on Gram-negative bacteria, as the outer membrane disables the entry of this molecule to the site of action (Boziaris and Adams, 2001; Lee *et al*. 2003). Current results are in line with previous reports, which have hypothesised that this is attributable to the differences in the cell wall structures; antimicrobial compounds can penetrate through the cell wall of Gram-positive bacteria and attack the cytoplasmic membrane, leading to not only leakage of the cytoplasm but also cytoplasm coagulation (Gandhi and Chikindas, 2007; Enan *et al*., 2013).

The findings from this study (Chapter 3) confirm that the fermented BM1 had the lowest value of pH and the highest TA value in the experimental preparation. This might be due to the conversion of sugars in the BM through fermentation to organic acids at different levels which is presented in the literature review (Sodini *et al*., 2006). Additionally, the fermented BM1 and nisin at 9 and 12µg/ml

demonstrated the highest inhibitory activity against some food pathogenic bacteria and *Bacillus cereus* spores. Moreover, the fermented BM1 had an inhibitory activity equivalent to 9µg/ml of nisin. Compared with other chemical preservatives, 9µg/ml of nisin was equivalent to 600ppm of calcium propionate and 900ppm of potassium sorbate or vinegar.

Consequently, it could be more challenging to use the fermented BM1 and 9µg/ml nisin additive (as a reference) as an alternative to chemical preservatives for baked goods (e.g. for crumpet formulations), which might be suitable for increasing the quality, texture, colour changes and extending the shelf life of bread crumpets.

The study described in Chapter 4 revealed potential influence of the BM1 fermented with *Lc. lactis* subsp. *lactis* (FBM) and commercial nisin additive on the quality and shelf life of bread crumpets. The results of this experiment showed that the bread crumpets with FBM had lower pH values, higher TA values, lower water activity, lower firmness and higher springiness during the storage period, and it has effects on the pore size of the crumpets compared to the other treatments. The change in pH level of the bread crumpets with FBM may be the result of adding fermented BM with *Lc. lactis* (homo-fermentative LAB) to the batter, which might cause pH to decrease and increase acidity of the batter. This is due to organic acid production that has been demonstrated by several studies to date (Walker and Klaenhammer, 2003; Hutkins, 2006). However, the use of FM by *Lc. lactis* may have been responsible for the decreased firmness and delayed staling. This agreed with previous studies that showed LAB can be used as a starter culture in the bread industry, which can delay bread staling during storage period (Plessas *et al*., 2008).

Consequently, our findings showed that the bread samples with fermented BM and nisin had a microbial shelf life of 8 days, whereas the control and crumpet breads with non-fermented buttermilk (NFBM) had a microbial shelf life of 6 days based on microbial counts. This might be due to metabolites of *Lc. lactis* during fermentation of BM or adding nisin-produced by *Lc. lactis* to the crumpet formulation which have antimicrobial activity against food pathogenic and/or spoilage microorganisms as presented by several studies (Messens and De Vuyst, 2002; Cooksey, 2005). The reduction of *Bacillus* counts and delayed mould growth in crumpets with FBM is likely associated with the presence of organic acids produced by LAB, which is presented in previous studies (Lynch *et al*., 2014). Axel *et al*. (2015) reported that the mould free shelf life of the Quinoa breads containing *Lb. amylovorus* fermented sourdough (gluten-free sourdough bread) increased by 4 days compared to the non-acidified control and also improved bread quality with reduced staling.

Sensory evaluation is an important step for new product development, it measures consumer preference for a particular product (Guàrdia *et al*., 2006). In this case, there was no difference in sensory attribute acceptability of all treatments. BM fermented with *Lc. lactis* successfully improved the quality, delayed the staling and extended the shelf life of crumpet breads by reducing the total microbial counts, moulds and yeasts and growth of *Bacillus cereus*. However, it gives more opportunity to minimise the use of chemical additives for the preservation of bakery products. This work could lead to improvements of current methods used for the preparation of bakery products.

Two other chapters (Chapter 5 and 6) were focused on the role of LAB sourdough, which is a considerably more complex system. However, making

bread products using starter cultures isolated from sourdough might achieve optimum quality, delayed staling, palatability and shelf life.

The purpose of this study (Chapter 5) was to assess the biodiversity of LAB strains from sourdough collection using molecular method (16S rRNA PCR-DGGE). This might help to explore sourdough microflora, and use that information in relation to their role. The potential influence of isolated LAB from sourdoughs was also investigated, establishing that some of them had a high antimicrobial activity against food pathogenic microorganisms. Isolated LAB strains are known to be useful as a starter culture for preparing high quality sourdough products (De Vuyst *et al*., 2002). They can also be useful to achieve improved bread quality or to obtain “clean label” products as well as to improve shelf life of the bread products. Gene sequencing showed that *Lactobacillus* was the predominant genus in the studies of sourdoughs, in agreement with the other studies (Savic *et al*., 2013). Microbial interactions, type of flour, low and variable availability of nutrients, environmental stresses during processing, and changes in the technology can be some of the factors which affect the biochemical and physiological responses of LAB in sourdough (Şimşek *et al*., 2006; Serrazanetti *et al*., 2009).

Based on the DNA sequences, more of of the isolates of LAB from sourdoughs are *Lb. plantarum*, *Lb. brevis* and *Lb. rossiae* strains which is in agreement with Van der Meulen *et al*. (2007) which showed that the strains of *Lb. plantarum*, *Lb. fermentum*, *Lb. brevis*, *Lb. rossiae*, and *Lb. paraplantarum* were dominating some of the sourdough ecosystems.

Based on the results of 32 LAB strains, three different groups were proposed according to the pH and acidity. Some of the 32 LAB strains presented amylolytic (10) and proteolytic (12) activities. Additionally, from six LAB strains of high acid group, four and five of them had amylolytic and proteolytic activities respectively. These enzyme activities might be due to a decrease in pH and increase in acid production, which have been demonstrated by several studies which reported that the decrease in pH is linked with acid production and causes an increase in the protease and amylase activities on the flour (Thiele *et al*., 2002; Galle, 2013). Also Fadda *et al*. (2014) reported that organic acids produced by LAB affect the protein and starch fractions and reduce the pH which results in an increase in protease and amylase activities in the flour.

The inhibitory action of LAB could be due to the production of antimicrobial compounds based predominantly on organic acids (lactic acid and acetic acid) and bacteriocins. Hydrogen peroxide and diacetyl also have inhibitory activity which can restrict the growth of potential pathogenic and spoilage microorganisms (Holzapfel *et al*., 2001; Gerez *et al*., 2008). Five LAB strains presented a strong inhibitory activity against five food pathogenic bacteria using agar well diffusion and spot methods. The result is in agreement with the finding by Şimşek *et al*. (2006) who reported that LAB isolated from sourdough samples had different antimicrobial activities against food pathogenic and spoilage microorganisms, using agar spot and agar well diffusion methods. The knowledge about sourdough microflora is useful in selecting LAB strains as starter cultures. Safety, high antimicrobial activity, and technological efficacy have to be considered when selecting strains for the food fermentations. The findings from this study (Chapter 5) confirm that some LAB show potential as

starter cultures to ferment sourdough. However, further studies are needed to apply fermented intermediary products to be added to the bread formulations that might yield further insight into the strain's functionality and technological contributions to achieve optimum quality, palatability and shelf life of bread products.

Finally, the experiment (Chapter 6) was to investigate the potential effect of prepared sourdough fermentation with selected starter cultures in bread making where the dough samples were fermented at different concentrations of sourdough and different fermentation times and temperatures to improve safety, delayed staling, shelf life, quality changes and sensory properties of sourdough bread. The earliest production of fermented foods was based on spontaneous fermentation resulting from microflora development that were naturally present in the raw material. The quality of the end product was dependent on the microbial load and component of the raw material (Ray and Joshi, 2015). Moreover, the direct addition of selected starter cultures to raw materials has been an improvement in fermented food processing, which can result in a high degree of control over the fermentation process and standardisation of the end product (Leroy and De Vuyst, 2004). The latter can contribute to the microbial safety or offer one or more technological, nutritional, or health advantages. Examples are LAB that are able to produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins, useful enzymes, or those which have probiotic properties (Leroy and De Vuyst, 2004). This is regarded as a way of replacing chemical additives with natural compounds produced by LAB, and at the same time providing the consumer with new and

attractive food products. It also leads to a wider application area of starter cultures (Leroy and De Vuyst, 2004).

From 24 prepared sourdoughs that were tested (Chapter 6), the fermented sourdough with starter culture *Lb. plantarum* JCM1149 (SIN3) after five day back-slopping at 30ºC had a low pH value and high lactic acid production. The differences in the pH and acidity between fermented sourdoughs might be due to the use of different starter culture and flour spontaneous microflora (Robert *et al*., 2006; Banu *et al*. 2010). Gül *et al*. (2005) and Katina *et al*. (2009) reported that LAB decreased the pH and increased the acidity of fermented sourdough. The growth of LAB was also higher in SIN3 and this starter culture had a high antimicrobial activity as mentioned in Chapter 5. Diversity analysis of PCR-DGGE DNA fingerprinting revealed that the LAB diversity was higher in the sourdough samples when compared to the control dough.

In this study, SIN3 was used for making breads according to the high quality of this sourdough as a leavening agent. However, the bread dough was fermented by two different processes (fast fermented at 30ºC for 3h and slow fermented at 20ºC for 18h) to achieve the quality changes, sensory properties, shelf life and palatability of the bread product. The results showed that the bread with FFHSD had a lower pH, higher acidity and improved the quality attributes (e.g. increasing loaf volume and decreasing moisture and Aw). Sourdough addition for bread production has been found to have a positive effect on the bread volume and crumb structure as described by (Corsetti *et al*., 2000; Clarke *et al*., 2002). The drop in pH might be due to the activity of sourdough starter cultures in bread dough which influenced the nutritional properties of the bread products as reported by several studies (Poutanen *et al*., 2009; Gänzle and Gobbetti,

2013). The use of high amounts of sourdough to make the bread and fermentation temperature of the dough might result in higher acidity of the bread product which was pointed out by Plessas *et al*. (2008). The decrease of moisture and Aw of FFHSD might be due to the LAB activities, high amount of sourdough, fermentation time and use of different temperature for microbial growth. The low Aw may prevent the growth of pathogens on the outer surface of bread products. The bread with FFHSD had significantly lower hardness and delayed staling comparing to the other treatments in all cases of storage period. However, the lower hardness of the bread made with sourdough starters was pointed out when comparing to the bread made without sourdough as reported by Dal Bello *et al*. (2007) and Coda *et al*. (2010). The reduced staling might be due to the organic acids produced by LAB, which affect the protein and starch fractions and reduce the pH resulting in an increase in protease and amylase activities of the flour (Arendt *et al*., 2007; Fadda *et al*., 2014). In bakery products, bread staling has been responsible for huge economic losses to both the baking industry and the consumer (Gray and Bemiller, 2003).

Bread with SFCON and FFCON which were made without sourdough addition, had a shelf life of 6 and 8 days respectively, which might be due to the slow and fast fermentation time at different temperatures. However, the bread with FFLSD, FFHSD and SFHSD had a shelf life of 10 days when tested for the level of microbial counts (APC, LAB, mould and yeast colony counts, growth of *Bacillus cereus* and spore forming bacteria). The reason for this increase in the shelf life of sourdough bread is probably due to antimicrobial components produced by LAB during fermentation which reduce the growth of food pathogenic and spoilage microorganisms (Schnürer and Magnusson, 2005;

Moore *et al*., 2008). However, differences of shelf life among breads might be caused by the fermentation processes of bread-dough including time and temperature. The shelf life of bread products is limited by physicochemical changes including staling and microbiological spoilage (Leuschner *et al*., 1999).

Sensory evaluation is an important step in product development to evaluate the successfulness and acceptability of any product by consumers (Elia, 2011). In this case, breads from slow fermentation at 20ºC for 18h had lower sensory acceptability than breads with fast fermentation at 30ºC for 3h, including the control. The overall appearance and overall acceptability of breads with FFLSD and FFHSD were higher than the others which might be due to improvements in the sensory characteristics such as loaf volume, texture and flavour of breads as described by Rehman *et al*. (2006) and Nawaz *et al*. (2007). Differences in the temperature and fermentation time to produce the metabolic activity of microbes might be also affect the product, as previously studied by Meroth *et al*. (2003b) and Vogelmann and Hertel (2011).

Making bread with 18% of sourdough addition and fast fermentation by *Lb. plantarum* (SIN3) successfully resulted in a bread with improved texture, loaf volume and sensory acceptability. Furthermore, the bread had a shelf life over 10 days by reducing the growth of pathogenic and spoilage microorganisms as well as delaying staling during storage.

The main novel findings from this research demonstrate the usefulness and potential of starter cultures with antimicrobial properties for fermentation in intermediate ingredients (sourdough and buttermilk) and applications of these

intermediate ingredients on making bread products (sourdough and crumpet breads).

- BM fermented with *Lc. lactis* subsp. *lactis* which was used for crumpet breads as a model successfully improved the quality, safety and extended shelf life of crumpet breads for 8 days, which was longer than other treatments (6 days) by reducing the growth of total microbial counts, moulds and yeasts and growth of *Bacillus cereus* and delaying staling during storage.
- Some of LAB (Lb1, Lb11, and Lb14) were isolated from sourdough samples show potential as starter cultures based on acidification capacity, amylolytic and proteolytic activities and antimicrobial activity against food pathogenic microorganisms. However, these strains were applied to the fermented sourdough. There is also potential for fermented intermediary products to be added to the bread formulations to increase the safety, quality and texture, delay staling and increase the shelf life of bread.
- Making bread with 18% sourdough addition, which was fermented by *Lb. plantarum* (SIN3) as a leavening agent and at a fast fermentation process (30ºC for 3h) of the dough, was successfully affected to produce a bread with good qualities including improved texture, sensory acceptability, loaf volume and height of the bread product and decreased moisture and Aw. However, it was effective in increasing the safety and shelf life of the bread product over 10 days by reducing the growth of pathogenic and spoilage microorganisms as well as delaying staling during storage.

The approach of this study is likely to yield feasible improvements (e.g. acidification, decreasing moisture and Aw, texture, increasing safety, extending shelf life and delaying staling) of the current methods for the preparation of baking goods.

7.2 Future work

The following areas can be studied further:

- The action of selected starter cultures of LAB and their metabolic components on proteins embedded in the cytoplasmic membrane and on phospholipids in the membrane could be a focal area for future research. Further elucidation of these mechanisms against pathogenic and spoilage bacteria related to bakery product is important and would provide insights that may prove usefulness for technological applications. The use of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to investigate the mechanisms of action of selected starter cultures against cell membranes, and also more details can be obtained related to the alteration in cell morphology.
- Regarding the antibacterial activity of selected starter cultures of LAB against pathogenic bacterial strains, the research is needed to increase knowledge regarding the effect of selected starter cultures and their metabolites on the mould growth in bread products by testing their antifungal activities against some specific moulds, which can grow on bread such as *Aspergillus*, *Eurotium*, *Penicillium*, *Wallemia sabi*.

- Study the prevalent organic and volatile compounds in sourdough and their contribution to bread flavour and aroma using different techniques including HPLC and gas chromatography–mass spectrometry to identify the quantity and identity of the compounds.
- Study the ability of selected starter cultures fermentability in treated (sterile) and non-treated commercially available flour in order to evaluate their performance in both the absence and presence of microflora for their sourdough fermentation ability.
- Exploration of the application of probiotic heath-resistant sporeformers that could survive the fermentation and baking process
- Study the microstructure of crumpet and sourdough bread using Cryo-SEM in order to investigate and visualise the influence of fermentation on the microstructure bread, starch granule gelatinization and gluten matrix and connect to the change in bread quality.
- Further investigation is needed in making sourdough using combination of leavening agents (selected starter cultures and addition of yeasts) and interactions between them in fermentation process, which may increase the quality and dough improvement. However, investigate their influence on the bread product including quality, sensory characteristics, bread improvement and shelf life.
- Bread is one of the highly consumed products in Kurdistan regional of Iraq. Investigating the potential of selected LAB and their metabolites in fermentation of different types of Kurdish bread need further research. This is particularly when microbial spoilage is the most and fastest way of shortening the shelf life.

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Appendices

Appendix A

Sensory evaluation of crumpet bread that added with natural preservative nisin and fermented buttermilk

Assessor number: . . . . . Sample code: . . . . . .

Please evaluate and indicate your opinion about each attribute by ticking (X) in a suitable box for each attribute. Please make sure that your results are placed under the correct code.

Sensory attributes	1 dislike extremely	5 neither like or dislike	9 Like extremely
Overall appearance	1 2 3 4 5 6 7 8 9		
Aroma	1 2 3 4 5 6 7 8 9		
Texture	1 2 3 4 5 6 7 8 9		
Flavour	1 2 3 4 5 6 7 8 9		
Acidity	1 2 3 4 5 6 7 8 9		
Overall acceptability	1 2 3 4 5 6 7 8 9		

Additional comments:

Appendix B

Research training and development

1. Modules training and development

1.1 BIO5124 (Postgraduate Research Skills and Methods) 3rd Oct. – 12th Dec. 2012.

No.	Date	Module	Facilitator	Venue
1	03/10/2012	Research ethics and methodology in science	Dr John Eddison	Babbage building
2	03/10/2012	Research ethics and methodology in science	Dr John Eddison	Babbage building
3	05/10/2012	Field safety and risk assessment	Dr Paul Ramsay	SCB 001
4	17/10/2012	Good laboratory practice	Dr Jha AN	Babbage building
5	17/10/2012	Laboratory safety and risk assessment	Dr Andy Foey	Babbage building
6	05/10/2012	Written communication	Dr Paul Ramsay	Babbage building
7	24/10/2012	Field safety and risk assessment	Dr Paul Ramsay	Babbage building
8	24/10/2012	Writing scientific papers	Dr Paul Ramsay	Babbage building
9	25/10/2012	Writing and publishing papers	Dr Paul Ramsay	Babbage building
10	25/10/2012	Working with literature: e-resources & adv. searching	Titley G	Babbage building
11	21/11/2012	Biostatistics I	Dr Miguel Franco	Babbage building
12	28/11/2012	Oral presentations	Dr Piero Calosi	Babbage building
14	28/11/2012	Biostatistics II and III	Dr Miguel Franco	Babbage building
15	05/12/2012	Project management and funding	Dr Ted Henry	Babbage building
16	05/12/2012	Poster presentations	Prof. Rod Blackshaw	Babbage building
17	12/12/2012	Public communication of science	Dr Maria Donkin	Babbage building
18	12/11/2012	Careers in biology	Ms Mandy Burns	Babbage building

1.2 BIO5102 (Principles and Applications of Electron Microscopy) 28th Sep. - 14th Dec. 2012. Dr Roy Moate

1.3 ENV5101 (Laboratory Teaching Methods and Practice) 25th Oct. – 07th Dec. 2012. Dr David Harwood

1.4 DIET107 (Food safety and quality 1 practical session and 6 lectures) November- December 2012, Dr Victor Kuri and Dr Jane Beal.

2. Postgraduate Research Skills and Training Sessions

No.	Date	Training skills	Facilitator	Venue
1	19/04/2012	Fisher trip to Science World	Michele Kiernan	London, UK
2	03/05/2012	Research Owning and Using	Graham Titley	Portland square Plymouth University
3	10/05/2012	What is La Tex		Rolle building Plymouth University
4	30/05/2012	Careers: Simulated Assessment Centres	Sara and Jolia	Babbage building Plymouth University
5	06/06/2012	Introduction to EndNote	Subject Librarians	Babbage building Plymouth University
6	12/06/2012	Introduction to applying for research funding	John Martin	Roland Levinsky Plymouth University
7	18/06/2012	Session Research Methodology	Dr Martin Coath	Babbage building Plymouth University
8	31/10/2012	Keeping Laboratory Records	Dr Rich Boden	Rolle building Plymouth University
9	22/06/2013	Transfer Process	Mick Fuller	Roland Levinsky Plymouth University
10	01/05/2013	Writing up and completing the thesis	Stacey DeAmicis; Joe Allison and Carolyn Gentle	Fitzory building Plymouth University
11	10/03/2016	Preparing to submit on pearl including copyright and open access	Kate Russel	Babbage building Plymouth University

3. Conferences and courses attended

No.	Date	Event	Venue
1	06/06/2012	Postgraduate conference for computing: Application and theory PCCAT2012	Plymouth University - United Kingdom
2	26/06/2012	The post graduate Society Annual Conference	Plymouth University - United Kingdom
3	04/07/2012	CARS Postgraduate Symposium	Duchy College - United Kingdom
4	21/11/2012	The post graduate Society Conference Series	Plymouth University - United Kingdom
5	10/12/2012	CARS Postgraduate Symposium	Plymouth University - United Kingdom
6	21/05/2013	Microstructure for food products conference	University of Reading, United Kingdom
7	18/06/2013	The post graduate Society Conference Series	Plymouth University - United Kingdom
8	15/09/2013	Bread Making Classes (Super Sourdough)	Red Dog Bakery Black Torrington- Devon
9	11/11/2013	CARS Postgraduate Symposium	Duchy College - United Kingdom
10	17/06/2014	The Postgraduate Society Conference Series	Plymouth University - United Kingdom
11	19/11/2014	6th CARS Postgraduate Symposium	The Eden Project, Boldeva, Cornwall, UK
12	24/03/2015	The Postgraduate Society Conference Series	Plymouth University - United Kingdom
13	14-15 /05/2015	IFST conference	London
14	23/06/2015	The post graduate Society Conference Series	Plymouth University - United Kingdom
15	06/02/2016	Artisan Baking (one-day course)	Dartington / Totnes - United Kingdom
16	14-15 /04/2016	Spring conference on probiotics	University of Reading, United Kingdom
17	04-07 /07/2016	SfAM Summer Conference	Edinburgh, Scotland United Kingdom

4. Membership of Scientific Societies:

- Society of Applied Microbiology (*s*fam)
- Institute of Food Science and Technology (IFST)

Appendix C

Food Safety Certificate

Chartered Institute of Environmental Health

Level 2 Award in Food Safety in Catering

1 credit

Rebaz Aswad Mirza Koy

has successfully completed a programme of training and an assessment which concluded the course

Course Director

Chief Executive
Graham Jukes
Chartered Institute of Environmental Health

Examination Date: *30 November 2012*

CIEH recommends you refresh your training by *30 November 2015*

Centre number: *23168*

Certificate number: 8894583

Original Issue Date: *11 December 2012*

Issue number: 1

PASS

Qualification accreditation number – 500/5476/4
Accredited only for England, Wales and Northern Ireland

2PNCCERT

Ofqual

Llywodraeth Cymru
Welsh Assembly Government

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Rewarding Learning

Appendix D

Posters

Potential Application of Bacteriocin Producing *Lactococcus lactis* Subsp. *lactis*, and Fermentation Products to Increase the Shelf Life and Safety of Crumpets

Rebaz Koy, Daniel Merrifield and Victor Kuri
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rebaz.koy@plymouth.ac.uk

RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

- Lactic acid bacteria (LAB) are safe to use in fermented foods
- LAB play an important fermentative role contributing to:
  - prolonging shelf life
  - increasing safety
  - improving the nutritional value
  - sensory characteristics
- Bacteriocins are natural antibacterials produced by LAB and inhibit the growth of some foodborne pathogenic microorganisms
- Buttermilk (BM) is a dairy by-product of cheese and butter preparation
- BM has been widely used in the food industry and domestically worldwide
- Valuable contributions to food systems include: emulsifying capacity, flavour and a source of affordable protein

Objective:

The objective was to investigate the contribution of natural antibacterial on the shelf life of bread crumpets. It was hypothesised that fermented buttermilk containing bacteriocins and commercial nisin preparations could reduce pathogenic bacterial sporulation during storage

Crumpets
-leavened baked bread
-baked centrally
-retailed as ready to eat
-stored at room temperature

Methods:

- Four different types of fermented buttermilk (BM) products with *Lactococcus lactis* subsp. *lactis* and commercial nisin (3, 6, 9, 12 µg/ml) as reference were examined *in vitro* for antibacterial activity against some pathogenic bacteria
- Three brands of commercial buttermilk powder were prepared either by adjusting for the same sugar content, or solid content with a control, and a fourth buttermilk was churned from commercial cream as shown below

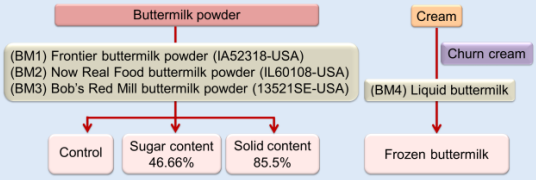

Figure 1: buttermilk preparation design

Characterising buttermilk samples and the commercial nisin

- pH and titratable acidity (TA) measurements for buttermilk products
- Agar diffusion bio-assay for activity of *Lc. lactis* subsp *lactis* fermented buttermilk products against pathogens
- Determination of the antimicrobial activities of buttermilks' supernatant and the commercial nisin against four foodborne pathogens (FBP)
- Assessment of nisin and fermented buttermilk activities against *Bacillus cereus* sporulation

Results:

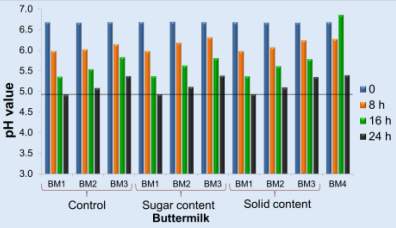

Figure 2: pH of buttermilk (BM1-BM4) prepared by three different methods and fermented with *Lc. lactis* subsp. *lactis* within 24 h at 30 °C

Table 1: Antibacterial activity of fermented buttermilk preparations against bacterial food pathogens using an agar well diffusion method

Fermented buttermilk		Target strains			
Preparation	Brand	*Bacillus cereus*	*E. coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Control	BM1	+++	++	++	+++
	BM2	++	++	+	++
	BM3	++	-	+	+
Fixed sugar content 46.7 %	BM1	+++	++	++	+++
	BM2	++	+	-	++
	BM3	++	–	+	+
Fixed solid content 85.5%	BM1	+++	++	++	+++
	BM2	++	+	-	+
	BM3	+	-	+	+
Churned buttermilk	BM4	+	-	++	-

Diameter of inhibition zone : (–) no inhibition zone, (+) weak (6 – 9 mm), (++) intermediate (10 – 13 mm) and (+++) strong (14 – 16 mm). Mean values from three replicates

Table 2: Microbial growth* (OD_{595}) at 24 h of incubation at 37°C, as affected by fermented buttermilks' supernatant or concentration of nisin

Antimicrobial Component	BM type	Conc. µg/ml	Target strains			
			Bacillus cereus	*E. coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Control		0	1.53±0.06 ^{a}	1.35±0.04 ^{a}	1.59±0.04 ^{a}	1.48±0.07 ^{a}
Fermented buttermilk type (%1)	BM1		0.44±0.04 ^{d}	0.55±0.03 ^{e}	0.47±0.02 ^{e}	0.45±0.02 ^{ef}
	BM2		0.56±0.02 ^{c}	0.58±0.02 ^{e}	0.59±0.02 ^{d}	0.53±0.01 ^{e}
	BM3		0.61±0.03 ^{c}	0.72±0.03 ^{d}	0.64±0.02 ^{d}	0.82±0.02 ^{c}
	BM4		0.84±0.03 ^{b}	1.09±0.01 ^{c}	0.78±0.02 ^{c}	1.21±0.03 ^{b}
Nisin suspension		3	1.48±0.05 ^{a}	1.21±0.04 ^{b}	1.26±0.03 ^{b}	1.17±0.03 ^{b}
		6	0.95±0.02 ^{b}	0.74±0.05 ^{d}	0.78±0.03 ^{c}	0.63±0.04 ^{d}
		9	0.53±0.03 ^{cd}	0.58±0.04 ^{e}	0.48±0.04 ^{e}	0.38±0.01 ^{f}
		12	0.15±0.01 ^{e}	0.22±0.02 ^{f}	0.19±0.01 ^{f}	0.14±0.01 ^{g}
			R^2 = 0.960	R^2 = 0.974	R^2 = 0.991	R^2 = 0.978

* means ± standard deviations (n=3).
$^{a-g}$ means in the same column with different superscripts are significantly different (P < 0.05)

Table 3: Determination of *Bacillus cereus* spores sensitivity* to commercial nisin and fermented buttermilk

number of spores	Control	Nisin concentration (µg/ml)				Fermented BM type (1%)			
		3	6	9	12	BM1	BM2	BM3	BM4
10^{-1}	+	+	+	-	-	-	+	+	+
10^{-2}	+	+	-	-	-	-	-	+	+
10^{-3}	+	+	-	-	-	-	-	-	-
10^{-4}	+	-	-	-	-	-	-	-	-
10^{-5}	+	-	-	-	-	-	-	-	-

* (+) growth of *Bacillus cereus* after spore incubation at 37 °C for 7 days, (-) no growth

Discussion and Conclusions:

BM1 produced the lowest pH at 8,16 and 24h in all the preparation methods. The TA value was also increased in BM1 (data not shown). BM1 also demonstrated the highest inhibitory activity against some FBP. This may be due to the its highest acidity value (Sodini *et al.*, 2006). To compare the effects of BMs to different nisin concentrations against FBP, BM1 had an inhibitory activity equivalent to 9 µg/ml of nisin. Finally, BM1 has the same inhibitory activity as nisin at 9 and 12 mg/ml against *Bacillus cereus* spores.
In conclusion, the capability of different BM product types and preparations of and commercial nisin were examined *in vitro* against some pathogenic bacteria. BM1 had an inhibitory activity equivalent to 9 µg/ml of nisin. These fermented ingredients could be suitable to add to crumpet formulations.

Future work:

Fermented buttermilk and the commercial nisin will be selected for use as bio-preservatives for crumpet bread to tests for the assessment of safety, shelf life and microbiology. In addition, sensory and physical characteristic qualities (i.e. hardness, springiness) of crumpets will be assessed instrumentally and by using sensory panel evaluations to determine the acceptability of the developed product.
Further studies will include *in situ* antibacterial and probiotic production in breads to include sourdough fermentations

Acknowledgments:

Special thanks to my sponsor- Ministry of Higher Education, Kurdistan Regional Government for financial support

Thanks to Mr. Calvin Hopkins for proof-reading the poster

References:

SODINI, I., Mortin, P., Olabi, A. & JIMENEZ-FLORES, R. 2006. Compositional and functional properties of buttermilk: A comparison between sweet-sour and whey buttermilk. *Journal of Dairy Science*, 89, 225-236.

Effects of bacteriocin produced by *Lactococcus lactis* subsp. *lactis* and fermentation Buttermilk product on the shelf life and safety of crumpets

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RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

- Lactic acid bacteria (LAB) are safe to use in fermented foods.
- LAB play an important fermentative role contributing to: prolonging shelf life, increasing safety, improving the nutritional value and sensory characteristics
- Bacteriocins are natural antibacterials produced by LAB and inhibit the growth of some foodborne pathogenic microorganisms
- Buttermilk (BM) is a dairy by-product of cheese and butter preparation
- BM has been widely used in the food industry and domestically worldwide

Objective:

To investigate the contribution of natural antibacterial on the shelf life of bread crumpets. It was hypothesised that fermented buttermilk containing bacteriocins and commercial nisin preparations could reduce pathogenic bacterial sporulation during storage

Experimental Design:

T1= control group (crumpets without additive),
T2= crumpets with added natural preservative nisin,
T3= non-inoculated crumpets by using buttermilk
T4= inoculated crumpets with *Lc. lactis* subsp. *lactis* (fermented buttermilk)

Characteristics studied:

- Measuring pH value
- Measuring total titratable acidity (TTA)
- image analysis of bread samples via image J software
- Shelf life determination of bread crumpets:
  - Total viable bacteria
  - Examination of *Bacillus cereus*
  - Mold and yeast count

Results:

Table 1: pH values[1] of bread crumpets at 0, 2, 4, 6 and 8th days of storage period at room temperature

Treatment[2]	Time (days)				
	0	2	4	6	8
T1	6.26±0.01 ^{aA}	6.25±0.01 ^{aA}	6.22±0.01 ^{aAB}	6.19±0.01 ^{aB}	ND[3]
T2	6.19±0.01 ^{bA}	6.17±0.01 ^{bA}	6.16±0.01 ^{bA}	6.15±0.01 ^{bAB}	6.11±0.01 ^{aB}
T3	6.22±0.01 ^{abA}	6.20±0.01 ^{bAB}	6.27±0.01 ^{bBC}	6.15±0.01 ^{bC}	ND
T4	5.63±0.02 ^{cA}	5.63±0.005 ^{cA}	5.61±0.005 ^{cAB}	5.61±0.01 ^{cAB}	5.59±0.01 ^{bB}

[1] Results are mean values from three replicates ± standard deviations. $^{a-d}$ Means in the same column with different superscripts are significantly different ($P < 0.05$). $^{A-D}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)
[2] T1: Control, T2: Natural preservative nisin, T3: Non-inoculated buttermilk and T4: inoculated buttermilk
[3] ND: no determined

Figure 1: Illustration of how the software Image J uses contrast in the image to find the edges of pores and defines the regions representing voids before measuring their areas

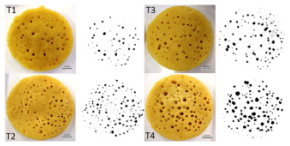

Table 2: Total viable count (TVC) and moulds and yeasts counts of bread crumpets at 0, 2, 4, 6 and 8th days of storage period at room temperature (Log_{10} CFU mL^{-1})

Microbes	Treatment	Time (days)				
		0	2	4	6	8
TVC	T1	4.11±0.03 ^{aD}	5.12±0.02 ^{aC}	5.32±0.01 ^{aB}	5.97±0.05 ^{aA}	ND[3]
	T2	3.92±0.02 ^{bE}	4.32±0.01 ^{cD}	4.96±0.03 ^{bC}	5.35±0.01 ^{bAB}	5.83±0.03 ^{aA}
	T3	4.16±0.03 ^{aD}	5.04±0.02 ^{bC}	5.27±0.01 ^{aB}	5.92±0.05 ^{aA}	ND
	T4	3.81±0.05 ^{cE}	4.27±0.01 ^{dD}	4.83±0.04 ^{dC}	5.05±0.03 ^{cB}	5.73±0.02 ^{bA}
moulds and yeasts counts	T1	0	3.58±0.06 ^{aC}	3.91±0.01 ^{aB}	4.31±0.02 ^{aA}	ND[3]
	T2	0	0	2.91±0.03 ^{bC}	3.57±0.04 ^{bAB}	4.11±0.01 ^{aA}
	T3	0	3.52±0.03 ^{aC}	3.85±0.01 ^{aB}	4.26±0.02 ^{aA}	ND
	T4	0	0	2.73±0.04 ^{cC}	3.53±0.03 ^{bB}	4.04±0.02 ^{bA}

Table 3: growth of *Bacillus cereus* of bread crumpets at 6, 7 and 8th days of storage period at room temperature (Log_{10} CFU mL^{-1})

Treatment	Time (days)		
	6	7	8
T1	2.77±0.05 ^{aB}	3.28±0.03 ^{aA}	ND[3]
T2	0	2.68±0.04 ^{bB}	2.94±0.03 ^{aA}
T3	2.67±0.05 ^{aB}	3.25±0.01 ^{aA}	ND
T4	0	2.54±0.04 ^{cB}	2.63±0.04 ^{bA}

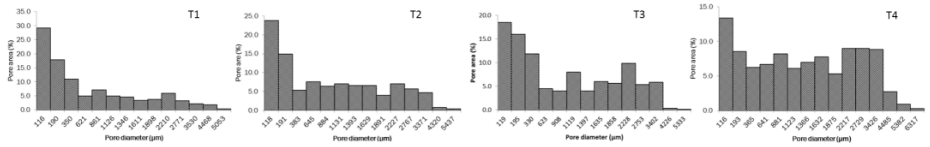

Figure 2: Pore size distributions obtained using image analysis of bread crumpets with added natural preservative nisin and fermented buttermilk product

Discussion and conclusions :

- The pH of T4 was lower and TTA was higher than the other treatments (data of TTA not shown). This result is in agreement with those found by Katina, *et al.* (2009).
- T2 and T4 increased the pores' size of bread crumpets in comparison with the control.
- T2 and T4 had a shelf life of 8 days. However, the other treatments had a shelf life of 6 days when tested for total viable count, mould and yeast colony counts and growth of *Bacillus cereus*. The results are in agreement with the results of Ogunbanwo, *et al.* (2008).

In conclusion, the fermented BM with *Lc. lactis* subsp. *lactis* decreased the pH value and increased TTA value of bread crumpets in comparison with the other treatments. The nisin additive and fermented BM had effects on the pores size of bread crumpets and had a shelf life of 8 days in comparison with the control.

Acknowledgments:

Special thanks to my sponsor - Ministry of Higher Education, Kurdistan Regional Government for financial support

References:

KATINA, K., MAINA, N. H., JUVONEN, R., FLANDER, L., JOHANSSON, L., VIRKKI, L., TENKANEN, M. & LAITILA, A. 2009. In situ production and analysis of *Weissella confuse* dextran in wheat sourdough. *Food microbiology*, 26, 734-743.
OGUNBANWO, S., ADEBAYO, A., AYODELE, M., OKANLAWON, B. & EDEMA, M. 2008. Effects of lactic acid bacteria and *Saccharomyces cerevisae* co-cultures used as starters on the nutritional contents and shelf life of cassava-wheat bread. *Journal of Applied Biosciences*, 12, 612-622.

The shelf life and safety of bread crumpets using fermented buttermilk and bacteriocins produced by *Lactococus lactis* subsp. *lactis*

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RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:
- Lactic acid bacteria (LAB) are safe to use in fermented foods
- LAB play an important fermentative role contributing to: prolonging shelf life, increasing safety, improving the nutritional value and sensory characteristics
- Bacteriocins are natural antibacterials produced by LAB and inhibit the growth of some foodborne pathogenic microorganisms
- Buttermilk (BM) is a dairy by-product of cheese and butter preparation
- BM has been widely used in the food industry and domestically worldwide

Objective:
To extend shelf life of bread crumpets through addition of a natural preservative and to compare commercial nisin and fermented buttermilk (BM) for their potential to reduce pathogenic bacterial sporulation on the product during storage at room temperature

Methods: **(Experiment Design)**
- control group (crumpets No additive, skimmed milk instead buttermilk)
- crumpets with added natural preservative nisin
- non-inoculated crumpets by using buttermilk
- inoculated crumpets with *Lc. lactis* subsp. *lactis* (fermented buttermilk)

Characteristics studied:
- pH and total titratable acidity (TTA)
- Texture: Firmness and springiness
- Colour
- Image analysis of bread samples via image J software
- Microbial shelf life determination
  - Total viable bacteria - *Bacillus cereus* - Mold and yeast

Results:

Table 1: pH* evolution over 8 days of storage of crumpets at room temperature

Treatment	Time (days)				
	0	2	4	6	8
Co-Control crumpets	6.26±0.01 ^{aA}	6.25±0.01 ^{aA}	6.22±0.01 ^{aAB}	6.19±0.01 ^{aB}	-
Nis-Crumpets with nisin	6.19±0.01 ^{bA}	6.17±0.01 ^{bA}	6.16±0.01 ^{bA}	6.15±0.01 ^{bAB}	6.11±0.01 ^{aB}
BM-Crumpets with Non-fermented BM	6.22±0.01 ^{abA}	6.20±0.01 ^{bAB}	6.27±0.01 ^{bBC}	6.15±0.01 ^{bC}	-
FBM-Crumpets with fermented BM	5.63±0.02 ^{cA}	5.63±0.005 ^{cA}	5.61±0.005 ^{cAB}	5.61±0.01 ^{cAB}	5.59±0.01 ^{bB}

*Mean values (n=3)±standard deviation. $^{a-d}$ Means in the same column with different superscripts are significantly different ($P < 0.05$). $^{A-D}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)
-: no determined

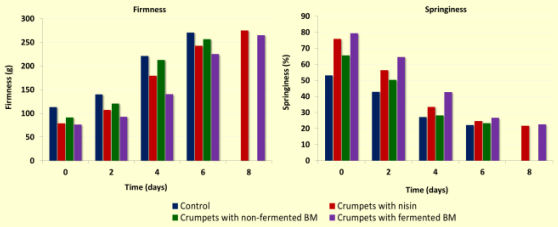


Figure 1: Firmness and springiness of bread crumpets over 8 days of storage at room temperature

Table 2: Total viable counts (TVC) and moulds and yeasts counts of bread crumpets over 8 days of storage at room temperature (*Log_{10} CFU mL^{-1})

Microbes	Treatment	Time (days)				
		0	2	4	6	8
TVC	Control	4.11±0.03 ^{aD}	5.12±0.02 ^{aC}	5.32±0.01 ^{aB}	5.97±0.05 ^{aA}	-
	Nisin	3.92±0.02 ^{bE}	4.32±0.01 ^{cD}	4.96±0.03 ^{bC}	5.35±0.01 ^{bAB}	5.83±0.03 ^{aA}
	Non-fermented BM	4.16±0.03 ^{aD}	5.04±0.02 ^{bC}	5.27±0.01 ^{aB}	5.92±0.05 ^{aA}	-
	Fermented BM	3.81±0.05 ^{cE}	4.27±0.01 ^{dD}	4.83±0.04 ^{dC}	5.05±0.03 ^{cB}	5.73±0.02 ^{bA}
moulds and yeasts	Control		3.58±0.06 ^{aC}	3.91±0.01 ^{aB}	4.31±0.02 ^{aA}	-
	Nisin			2.91±0.03 ^{bC}	3.57±0.04 ^{bAB}	4.11±0.01 ^{aA}
	Non-fermented BM		3.52±0.03 ^{aC}	3.85±0.01 ^{aB}	4.26±0.02 ^{aA}	-
	Fermented BM			2.73±0.04 ^{cC}	3.53±0.03 ^{bB}	4.04±0.02 ^{bA}

*Mean values (n=3)±standard deviation. $^{a-d}$ Means in the same column with different superscripts are significantly different ($P < 0.05$). $^{A-D}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)
-: no determined

Table 3: growth of *Bacillus cereus* of bread crumpets over 8 days of storage at room temperature (*Log_{10} CFU mL^{-1})

Treatment	Time (days)		
	6	7	8
Control	2.77±0.05 ^{aB}	3.28±0.03 ^{aA}	-
Nisin		2.68±0.04 ^{bB}	2.94±0.03 ^{aA}
Non-fermented BM	2.67±0.05 ^{aB}	3.25±0.01 ^{aA}	-
Fermented BM		2.54±0.04 ^{cB}	2.63±0.04 ^{bA}

*Mean values (n=3)±standard deviation. $^{a-d}$ Means in the same column with different superscripts are significantly different ($P < 0.05$). $^{A-D}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)
-: no determined

Figure 2: Whiteness crust top and crumb colour of bread crumpets with added natural preservative nisin and fermented buttermilk product. Different letters within treatments are significantly difference

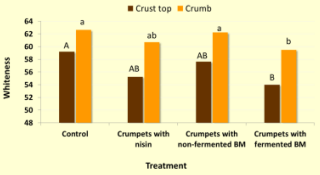
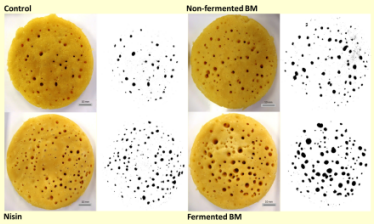


Figure 3: Rendered contrast images showing the edges of pores and defined void regions for area measurement with Image J software



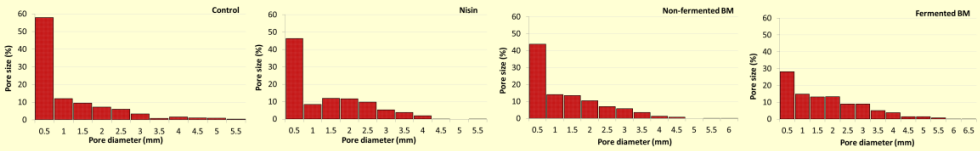


Figure 4: Pore size distributions obtained using image analysis of bread crumpets with added natural preservative nisin and fermented buttermilk product

Discussion and conclusions:
- the pH value and firmness of bread crumpets with fermented BM were lower and the TTA and springiness were higher than the other treatments (data of TTA not shown). Firmness was increased and springiness was decreased in all treatments during storage at room temperature. This result is in agreement with those found by Katina *et al.* (2009).
- bread crumpets with fermented BM increased the pores' size and changed the colour in comparison with the control.
- bread crumpets with nisin and fermented BM had a shelf life of 8 days. However, the other treatments had a shelf life of 6 days when tested for total viable count, mould and yeast colony counts and growth of *Bacillus cereus*. The results are in agreement with the results of Ogunbanwo, *et al.* (2008) who reported that Bread produced using LAB had a shelf life of 8-12 days, while 4 days for Control.

In conclusion, the fermented BM with *Lc. lactis* subsp. *lactis* decreased the pH value and increased TTA value of bread crumpets in comparison with the other treatments. firmness of bread crumpets with fermented BM was lower and springiness was higher than the other treatments. The nisin additive and fermented BM had effects on the pores size of bread crumpets and had a shelf life of 8 days in comparison with the control.

Acknowledgments:
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References

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Shelf life and safety of crumpets - Effects of the use of nisin and buttermilk fermented with *Lactococcus lactis* subsp. *lactis*

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Introduction:

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Objective:

To extend shelf life of bread crumpets by the addition of a natural preservative and to compare commercial nisin and fermented buttermilk (BM) for reductions of pathogenic bacterial sporulation on the product during storage at room temperature

Experiment Design	Characteristics studied
• **Con**- control crumpets -No additives, skimmed milk instead of buttermilk • **Nis**- with natural preservative nisin • **Nbm**- with buttermilk (non-inoculated) • **Fbm** –with fermented buttermilk (inoculated with *Lc. lactis* subsp. *lactis*)	pH and total titratable acidity (TTA) Microbial shelf life determination: • Total aerobic mesophilic bacteria (TAMB) • Mould and yeast • *Bacillus cereus* Crumpet quality: • Colour, • Texture (Firmness and springiness) • pore size (image analysis)

Results

Table 1: Total aerobic mesophilic bacteria (TAMB) and moulds and yeasts counts of bread crumpets over 8 days of storage at room temperature ($^{*}Log_{10}$ CFU mL^{-1})

Microbial groups	Treatment	Time (days) 0	2	4	6	8
TAMB	Control	4.11±0.03 ^{aD}	5.12±0.02 ^{aC}	5.32±0.01 ^{aB}	5.97±0.05 ^{aA}	
	Nisin	3.92±0.02 ^{bE}	4.32±0.01 ^{cD}	4.96±0.03 ^{bC}	5.35±0.01 ^{bAB}	5.83±0.03 ^{aA}
	Non-fermented BM	4.16±0.03 ^{aD}	5.04±0.02 ^{bC}	5.27±0.01 ^{aB}	5.92±0.05 ^{aA}	
	Fermented BM	3.81±0.05 ^{cE}	4.27±0.01 ^{dD}	4.83±0.04 ^{dC}	5.05±0.03 ^{cB}	5.73±0.02 ^{bA}
moulds and yeasts	Control	nd	3.58±0.06 ^{aC}	3.91±0.01 ^{aB}	4.31±0.02 ^{aA}	
	Nisin	nd	nd	2.91±0.03 ^{bC}	3.57±0.04 ^{bAB}	4.11±0.01 ^{aA}
	Non-fermented BM	nd	3.52±0.03 ^{aC}	3.85±0.01 ^{aB}	4.26±0.02 ^{aA}	
	Fermented BM	nd	nd	2.73±0.04 ^{cC}	3.53±0.03 ^{bB}	4.04±0.02 ^{bA}

*Mean values (n=3) ± standard deviation
nd- not detected
$^{a-d}$ Means in the same column with different superscripts are significantly different ($P < 0.05$)
$^{A-D}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 2: Growth of *Bacillus cereus* of bread crumpets over 8 days of storage at room temperature ($^{*}Log_{10}$ CFU mL^{-1})

Treatment	Time (days) 5	6	7	8
Control	nd	2.77±0.05 ^{aB}	3.28±0.03 ^{aA}	
Nisin	nd	nd	2.68±0.04 ^{bB}	2.94±0.03 ^{aA}
Non-fermented BM	nd	2.67±0.05 ^{aB}	3.25±0.01 ^{aA}	
Fermented BM	nd	nd	2.54±0.04 ^{cB}	2.63±0.04 ^{bA}

See footnote for Table 1

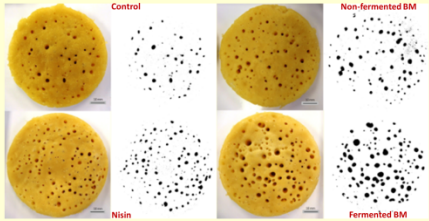

Figure 1: Rendered contrast images showing the edges of pores and defined void regions for area measurement with Image J software

Table 3: pH* evolution over 8 days of storage of crumpets at room temperature

Treatment	Time (days) 0	2	4	6	8
Control	6.26±0.01 ^{aA}	6.25±0.01 ^{aA}	6.22±0.01 ^{aAB}	6.19±0.01 ^{aB}	
Nisin	6.19±0.01 ^{bA}	6.17±0.01 ^{bA}	6.16±0.01 ^{bA}	6.15±0.01 ^{bAB}	6.11±0.01 ^{aB}
Non-fermented BM	6.22±0.01 ^{abA}	6.20±0.01 ^{bAB}	6.27±0.01 ^{bBC}	6.15±0.01 ^{bC}	
Fermented BM	5.63±0.02 ^{cA}	5.63±0.005 ^{cA}	5.61±0.005 ^{cAB}	5.61±0.01 ^{cAB}	5.59±0.01 ^{bB}

*Mean values (n=3) ± standard deviation. nd: not detected
$^{a-c}$ Means in the same column with different superscripts are significantly different ($P < 0.05$)
$^{A-C}$ Means in the same row with different superscripts are significantly different ($P < 0.05$)

Discussion and conclusions:

- the pH value and firmness of bread crumpets with fermented BM were lower and the TTA and springiness were higher than the other treatments (data of TTA not shown). Firmness was increased and springiness was decreased in all treatments during storage at room temperature.
- 71.85% of the pores size had diameter above 1 mm in bread crumpets with fermented BM, while 42% in the control bread.
- bread crumpets with nisin and fermented BM had a shelf life of 8 days. However, the other treatments had a shelf life of 6 days when tested for TAMB, mould and yeast colony counts and growth of *Bacillus cereus*.

In conclusion, the fermented BM with *Lc. lactis* subsp. *lactis* decreased the pH value and increased TTA value of bread crumpets in comparison with the other treatments. firmness of bread crumpets with fermented BM was lower and springiness was higher than the other treatments. Bread crumpets with fermented BM increased the pores' size and had a shelf life of 8 days in comparison with the control.

Quality of bread crumpets were studied:

- Fermentation (BM) increased the firmness and decreased the springiness of bread crumpets, possibly also linked to the increased pore size
- While microbiological changes were observed for all samples over 8 days, the texture deteriorated significantly with increases in firmness and reduction of springiness after 2 days
- Whiteness was lower for bread crumpets with fermented BM

Acknowledgments:
Special thanks to Ministry of Higher Education, Kurdistan Regional Government for financial support

Fermented dough lactic acid bacteria diversity – potential use as starter

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RESEARCH WITH PLYMOUTH UNIVERSITY

Introduction:

- Sourdough is a mixture of flour and water fermented by lactic acid bacteria (LAB) where yeast is also present.
- During sourdough fermentation, a range of LAB can produce metabolites which could influence the texture, prolonging shelf life, increasing safety, improving the nutritional value and sensory characteristics of bread

Objective:
To assess the diversity of LAB strains isolated from sourdough, considering their usefulness for making bread with acceptable properties including shelf life

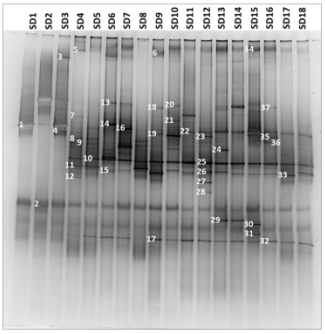
Methods:

- Collection of local and commercial sourdough samples (n=18)
- The diversity of microflora on the samples was analysed using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis, followed by statistical cluster analysis of the banding patterns.
- In total 34 strains were isolated from sourdough samples and identified by using biochemical methods and PCR and sequencing analysis, and some phenotypic tests.
- pH and titratable acidity (TA) for the sourdough samples and LAB isolates
- Determination of Proteolytic and Amylolytic enzymes activity for LAB isolate
- Antimicrobial activity of LAB isolates against some indicators bacteria using:
  - agar well diffusion method - agar spot method

Table 1: Sourdough samples description

Labels	Description	Acidity
SD1	Starter culture-(SC) Florapan L62K	Medium
SD2	SC-Florapan L73K	Medium
SD3	SC-Florapan LA4K	Medium
SD4	Wholemeal rye flour	Medium
SD5	Light rye flour	Law
SD6	White strong flour	High
SD7	Organic whole rye flour	Medium
SD8	Organic rye flour	High
SD9	San Francisco starter	Law
SD10	White strong flour	Medium
SD11	Organic white bread flour	Medium
SD12	Sourdough and rye flour	Medium
SD13	Organic white flour	Medium
SD14	Unbleached all-purpose flour	Medium
SD15	wholegrain rye flour	Medium
SD16	Hard white flour with sourdough culture	Medium
SD17	White strong flour	Law
SD18	San-Francisco sourdough bread	-

Results:



Numbers on bands correspond to identity of the bacterial strains determined by sequencing:
(1,3,15) *Lb brevis*
(2,4) *Lb. plantarum*
(5,8,9,11) *Lb. sanfranciscensis*
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(14) *Lb. farciminis*
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(16) *Lb.senmaizukei*
(13) *Lb. kimchicus*
(19) Lb. camelliae
(20) *Lb. alimentarius*
(21) *Lb. songhuajiangensis*
(24) *Lb. mindensis*
(26) *Lb. pontis*
(27,28) *Lb. reuteri*
(33) *Pediococcus parvulus*
(34) *Lb. backii*
(36) *Lb. kimchii*
others un-cutured *Lactobacillus*

Figure 1: DGGE fingerprints of sourdough samples. Number of bands (operative taxonomy units (OTUs) in each sample relates to diversity richness

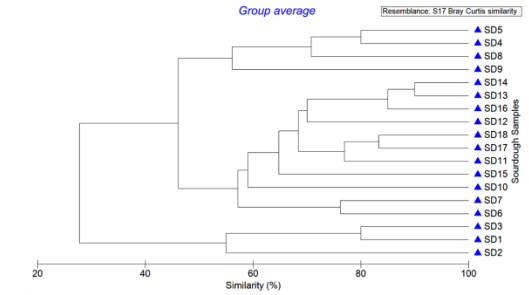

Figure 2: Cluster analysis based on the PCR-DGGE DNA fingerprints showing similarity (%) of bacterial communities between sourdough samples

Table 2: Identification of pure isolates from sourdough samples

Number of isolates	LAB isolates	Strain	Max. Identity (NCBI BLAST)
13	Lb2, Lb3, Lb4, Lb5, Lb6, Lb9, Lb10, Lb12, Lb14, Lb18, Lb24, Lb30, Lb32	*Lb. plantarum*	99%
12	Lb1, Lb8, Lb13, Lb15, Lb19, Lb25, Lb27, Lb28, Lb29, Lb31 Lb33, Lb34	*Lb. brevis*	99%
5	Lb7, Lb11, Lb17, Lb21, Lb23	*Lb. rossiae*	99%
1	Lb22	*Lb. siliginis*	98%
1	Lb26	*Lb. farciminis*	99%
2	Ab16, Ab20	*Acetobacter pasteurianus*	99%

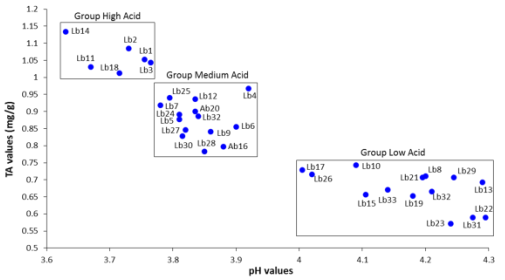

Figure 3: pH and TA values of isolated LAB from sourdough samples discriminating the cultures by acidity production level

Table 3: Antibacterial activity* of isolates of sourdough samples against indicator strains using an agar well diffusion method

Isolates	Source	Target strains				
		Bacillus cereus	*Bacillus subtilis*	*E. coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Lb1	SD1	+++	+++	+++	+++	+++
Lb2	SD2	+++	+++	+++	+++	++
Lb11	SD6	+++	+++	+++	+++	+++
Lb14	SD8	+++	+++	+++	+++	+++

*Diameter of inhibition zone: (++) intermediate (10 – 13 mm) and (+++) strong (14 – 16 mm)

Table 4: Proteolytic and amylolytic enzyme activities of LAB strains

enzyme activities	
Proteolytic activity	Amylolytic activity
Lb1, Lb2, Lb3, Lb5, Lb8, Lb11, Lb12, Lb14, Lb21, Lb22, Lb25, Lb29	Lb1, Lb2, Lb4, Lb11, Lb14, Lb15, Lb22, Lb29,Lb30, Lb32

Discussion and conclusions:

- Gene sequencing allowed identification of bacterial populations on sourdoughs, showing a predominance of the genus *Lactobacillus* in agreement with other studies (De Vuyst and Neysens, 2005) which was also related to diversity and richness indices.
- Ranges of pH and TA on sourdough samples were of 3.5 - 4.5 and 0.68 - 1.05 mg lactic acid per g respectively. Low pH and acidification would affect the sourdough characteristics including changes in enzyme activity. Good sugar fermenting strains, also happened to be hydrolytic enzyme producers, when tested in absence of sugar.
- Most strains were *Lb. plantarum* and *Lb. brevis*, while two strains were identified as *Acetobacter pasteurianus*.
- Some of the 34 strains presented amylolytic (10) and proteolytic (12) activities, but most were also good acid producers after 24 h of incubation at 30°C with pH 3.6 - 4.3 and lactic acid from 0.57 - 1.13 mg/g. Some strains of LAB had a strong inhibitory activity against five indicator strains on agar well diffusion and agar spot tests (data of agar spot method not shown). The performance as sourdough starters will be tested on to validate potential increase on the safety and shelf life of sourdough bread. Quality attributes of sourdough bread including stability, processability, and sensory properties will be evaluated.

The biodiversity of LAB isolate strains of sourdough samples was rich, but some *Lb.* species had a predominant role. The strong inhibitory activity of some LAB could be helpful as starter cultures for sourdough fermented breads to increase the safety and quality.

Acknowledgments:
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Reference:
De Vuyst L, Neysens P (2005): The sourdough microflora: biodiversity and metabolic interactions. Trends in Food Science & Technology, 16:1-3, p. 43-56.

Diversity of lactic acid bacteria from fermented dough – Potential use as sourdough bread starters

Rebaz Koy, Daniel Merrifield and Victor Kuri

rebaz.koy@plymouth.ac.uk School of Biological Sciences, Plymouth University, PL4 8AA, UK

RESEARCH WITH PLYMOUTH UNIVERSITY

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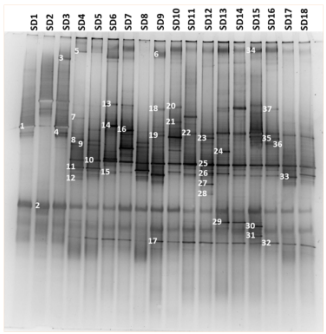

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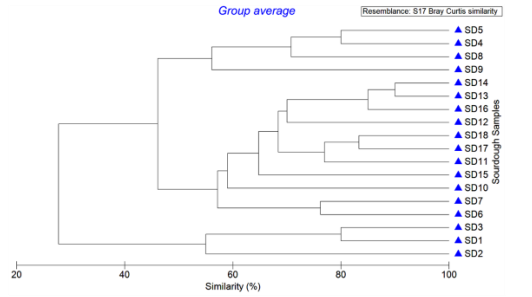

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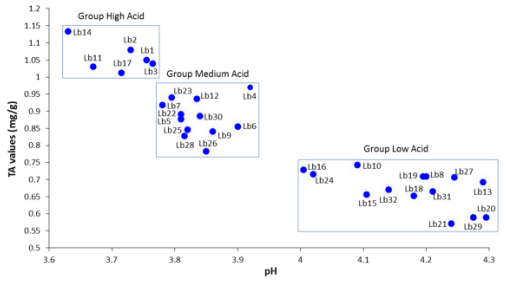

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Table 4: Proteolytic and amylolytic enzyme activities of LAB strains

Acidity groups	Enzyme activities	
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Medium acid	Lb5, Lb12, Lb23	Lb28, Lb30
Low acid	Lb8, Lb19, Lb20, Lb27	Lb15, Lb20, Lb27

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